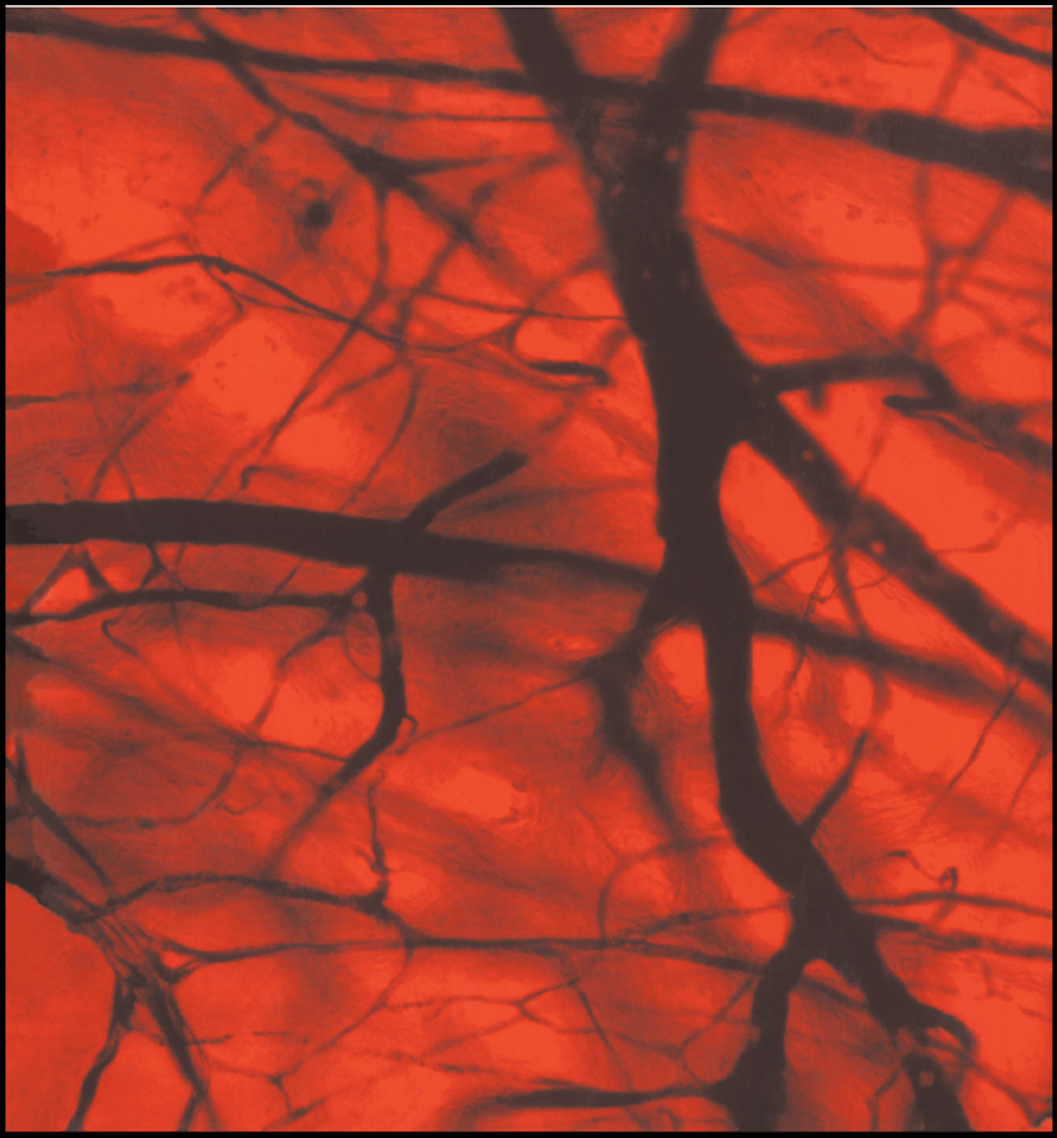
Genetics *of* Angiogenesis

J.B. Hoying (Ed)



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Preface

This book has the title "Genetics of Angiogenesis". In this case, the term genetics is being used in the broadest sense, beyond the traditional definition of inheritance, to address how genes give rise to blood vessel formation. With this book, the goal is not to provide a comprehensive review of the genes (or molecules) relevant to angiogenesis. Instead, the intent is to stimulate discussion of emerging concepts in angiogenesis describing the molecular and genetic programs, including how these programs themselves are modulated, regulating angiogenesis and complex vascular phenotypes.

Angiogenesis, the establishment of new blood vessels from an existing vasculature, is an essential component in a variety of vertebrate biological processes including development, growth, physiology, tissue repair and disease. Consequently, considerable research has been, and continues to be, directed at characterizing the many features and mechanisms of new vessel growth. Research to date has identified hundreds of genes that are believed to mediate angiogenesis. Although many genes are essential for new blood vessel growth, no single gene appears to be sufficient to produce a functional, vascular tube. Furthermore, investigation into the molecular biology of many of these genes and gene products is revealing a complex molecular milieu that gives rise to new vessel elements. As a consequence, traditional views of the "angiogenic factor" are being redefined and new molecular paradigms are being described. The next phase of angiogenesis research will have to begin examining the mechanisms by which these varied and complex systems are integrated to produce a functional vessel element.

Clinically, management of angiogenesis offers considerable potential in treating a number of diseases and conditions. Consequently, much of the impetus for understanding the mechanistic nature of angiogenesis has come from the study of disease and disease processes. However, angiogenesis can serve as a powerful model system to better understand complex, integrated biological systems. For example, during angiogenesis, vascular cells exhibit a high degree of phenotypic plasticity. They perform a variety of cellular activities leading to the formation of a new three-dimensional tube which is then organized into a network of other vascular tubes. Embedded in this process are gene expression programs to control the cellular activities, molecular control mechanisms to modulate the "execution" of these programs, cell-environment interactions that establish the tube phenotype, homeostatic regulatory loops which tie together the process with tissue and organ function, and other biological processes. Understanding how

angiogenesis happens will provide answers to many known, and yet-to-be defined, fundamental biological questions.

The book has been organized into four sections. Each section groups chapters pertinent to some aspect of angiogenesis genetics, emphasizing emerging knowledge in the represented topic. In the first section *Molecular Biology,* three chapters consider the molecules that participate in angiogenesis and the mechanisms by which angiogenesis is regulated. Chapter 1 surveys the heterogeneous classes of molecules that are important in angiogenesis and establishing vascular heterogeneity. Chapter 2 presents the emerging story of the molecular guidance system" for angiogenesis, the ephrin-Eph pathways. Chapter 3 addresses the complex biology of the unique angiopoietin family of angiogenesis molecules. The following section, *Molecular Genetics,* addresses detailed aspects of gene regulation in angiogenesis. Research into the regulatory features of angiogenesis-related gene expression and the coordination into effector programs is uncovering unique molecular activities and processes. Chapter 4 discusses the role of the ETS family of transcription factors in transcriptional regulation of angiogenesis genes. Chapter 5 introduces recent work uncovering mechanisms related to post-transcriptional regulation of the central angiogenesis factor, VEGF. Chapter 6 complements the discussions in Chapters 4 and 5 by examining the role of hypoxia and epigenetic influences on angiogenesis-related gene regulation. The third section, *Genetic Models,* presents three different model systems that are proving invaluable in determining the molecular physiology" of angiogenesis genes. Chapter 7 presents transgenic strategies for studying complex molecular contributions to angiogenesis. In Chapter 8, the power of mutagenesis screens for identifying genetic elements of angiogenesis is highlighted. Chapter 9 describes what we have learned from, perhaps the most classical of genetic models, that of inherited human disorders. The final section, *Genomics and Complexity,* introduces evolving ideas arising from recent genome-scale and combinatorial approaches at addressing systematic aspects of angiogenesis and the vasculature. Chapter 10 compares the genetic programs executed between embryonic angiogenesis and adult angiogenesis. Chapter 11 presents novel strategies by which vascular heterogeneity, and the molecular basis for determining heterogeneity, can be characterized and studied. Chapter 12 builds on these discussions and attempts to characterize the basis for complexity in angiogenesis.

Each chapter represents the ideas of its author(s) and is intended to stand alone. However, all of the chapters together should provide a continuity of topics related to angiogenesis and how it occurs. It is my hope that this book will stimulate further discussion into this intriguing biological process and provide some new insight into angiogenesis research.

James B.Hoying

1
Genetic determinants of endothelial cell heterogeneity

Takashi Minami and William C.Aird

1. Introduction

Endothelial cells line the inside of all blood vessels, playing a role in a multitude of physiological processes, including the control of cellular trafficking, the regulation of vasomotor tone, the maintenance of blood fluidity, and the growth of new blood vessels (Cines *et al.,* 1998). It is important to recognize that the structure and function of endothelial cells differ in time and space (Garlanda and Dejana, 1997; Gerritsen, 1987; Page *et al.,* 1992; Risau, 1995). As a general rule, endothelial cell phenotypes vary: 1) between different organs; 2) between different segments of the vascular loop within the same organ; and 3) between neighboring endothelial cells of the same organ and blood vessel type (Aird, 2001). For example, the von Willebrand factor gene is expressed predominantly within the endothelium of veins (Aird, 2001; Yamamoto *et al.,* 1998), tissue factor pathway inhibitor is a marker for microvascular endothelium (Osterud *et al.,* 1995), while thrombomodulin is expressed in the vasculature of all organs except the brain (Ishii *et al.,* 1986). Recent *in vivo* phage display studies have uncovered a wide array of genes that are expressed in specific vascular beds of normal organs and tumors (Rajotte *et al.,* 1998; Trepel *et al.,* 2000). While endothelial cell complexity and diversity have long been recognized, little is known about the underlying molecular mechanisms that mediate phenotypic heterogeneity of different endothelial cell populations.

Phenotypic diversity may arise from factors that are inherent in the cell or from signals residing in the extracellular environment (Stevens *et al.,* 2001). The genetic (or intrinsic) hypothesis predicts that organ-specific phenotypes are predetermined before they migrate from the mesoderm to specific vascular beds. Support for this model derives largely from fate mapping studies in vertebrate embryos, showing that endothelial cell precursors, or angioblasts, are capable of differentiating along several genetically pre-programmed lines. The environmental (or extrinsic) hypothesis holds that site-specific properties of endothelial cells are governed by local microenvironmental cues present within the

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resident tissue. Indeed, there is a growing appreciation for the importance of the local environment in determining endothelial cell phenotypes. The interaction between the microenvironment and the endothelial cell may involve soluble mediators, cell-cell communication, and the synthesis and organization of matrix proteins. In the final analysis, phenotypic heterogeneity of the endothelium is likely determined by a combination of genetic and environmental factors.

In keeping with the theme of this book, the current chapter focuses on the genetic determinants of endothelial cell heterogeneity. For an overview of the environmental determinants of endothelial cell structure and function, the reader is referred to a number of recent review papers (Cines *et al.,* 1998; Gerritsen and Bloor, 1993; Rosenberg and Aird, 1999).

2.
Genetic predeterminants of endothelial cell heterogeneity

2.1
Coronary artery versus endocardium

Retroviral cell lineage studies in the chick embryo have uncovered distinct embryonic origins for coronary endothelium and the endocardium (Mikawa and Fischman, 1992). The coronary endothelium, but not the endocardium, has been shown to derive from the proepicardial organ located in the dorsal mesocardium. Interestingly, these progenitor cells are multipotent, giving rise to endothelial cells, vascular smooth muscle cells and fibroblasts of the coronary vessel. The genetic programs that underlie the site-specific formation of blood vessels within the heart have yet to be elucidated.

2.2
Endothelial cell phenotypes in the lung

There is increasing evidence that endothelial cells from micro-and macrovascular segments of the lung derive from different origins during embryogenesis (deMello and Reid, 2000; deMello *et al.,* 1997; Stevens *et al.,* 2001). For example, microvascular endothelium in the lung is derived from blood islands through a process that involves vasculogenesis, whereas macrovascular endothelial cells originate from the pulmonary truncus by angiogenesis. Although these genetic programs may be important in specifying microvascular and macrovascular fates, terminal differentiation is likely to be critically dependent on microenvironmental cues.

2.3
Arteries versus veins

One of the most important events during vascular development is the specification of endothelial tubes as arteries and veins. Until recently, it was widely believed that the

phenotypic differences between arterial and venous endothelial cells were attributable to environmental factors, such as differences in hemodynamic forces, direction of blood flow, oxygen levels, subendothelial matrix and interactions with neighboring smooth muscle cells. This view has been challenged by the recent discovery of certain molecules that are specifically expressed in arterial or venous endothelial cells early during development before the onset of circulation. As a result of these observations, there is an intense interest in delineating the molecular basis for arterial-venous identity.

Ephrins. In the mouse, blood vessels that are fated to become arteries express ephrinB2, a member of a large class of transmembrane proteins (Adams *et al.,* 1999). This pattern persists in adulthood (Gale *et al.,* 2001; Shin *et al.,* 2001). In contrast, cells with a venous fate express EphB4, the proposed tyrosine kinase receptor for ephrinB2 (Wang *et al.,* 1998). The targeted disruption of the ephrinB2 gene leads to embryonic lethality at day E11 owing to a defect in both arterial and venous vessel remodeling (Wang *et al.,* 1998). Mice lacking EphB4 display a similar vascular phenotype (Gerety *et al.,* 1999). Taken together, these results suggest that the establishment of venous and arterial identity is a critical step in the development of the cardiovascular system and that ephrin signaling plays a central role in this process.

Notch signaling. The Notch signaling pathway, which consists of four Notch family receptors and five transmembrane ligands, has been implicated in blood vessel development. In zebrafish, Notch 3 is expressed in a pattern that is similar to that of ephrinB2, namely in the arterial endothelium (Lawson *et al.,* 2001). In E13.5 mouse embryos, Notch 1 is localized to the descending aorta, Notch 3 to the smooth muscle layer of the descending aorta and other arteries, and Notch 4 to arterial endothelium (Villa *et al.,* 2001). Expression of the Notch ligands, Jagged1 and Dll4, is restricted to arterial endothelium (Shutter *et al.,* 2000; Villa *et al.,* 2001). Taken together, these data suggest that Notch ligand-receptor interactions are involved in site-specific signal transduction.

The targeted disruption of Notch 1 resulted in abnormal vascular morphogenesis (Krebs *et al.,* 2000), while over-expression of a constitutively active form of Notch 4 within endothelial cells disrupted normal vascular development (Uyttendaele *et al.,* 2001). The targeted disruption of the Notch ligand, Jagged1, resulted in defects in the formation of blood vessels within the head and yolk sac, but not of the larger vessels (Xue *et al.,* 1999), adding further support for a vascular bed-specific role for this molecule. In zebrafish, the loss of Notch signaling results in molecular defects in arterial-venous differentiation, including the loss of artery-specific markers (i.e. ephrinB2) and the ectopic expression of venous markers (i.e. *flt4* and *rtkS*) within the dorsal aorta (Lawson *et al.,* 2001). Furthermore, the over-expression of Notch results in repression of venous cell fate (Lawson *et al.,* 2001). Together, these observations suggest that appropriate levels of and regulation of Notch signaling are critical for normal vascular development and arterial fate (Gridley, 2001).

Gridlock. Using lineage tracking in zebrafish embryos, Zhong *et al.* demonstrated that angioblast precursors in the lateral posterior mesoderm are fated to migrate to either the arterial or venous side of the circulation (Zhong *et al.,* 2001). The decision to enter an artery or vein was governed by *gridlock (grl),* an arteryrestricted gene that is expressed in

the lateral posterior mesoderm (Zhong *et al.,* 2000). The downregulation of *grl* resulted in a loss of arteries and an expansion of veins, changes that were associated with an induction of the venous marker EphB4 and a reduction in the arterial marker ephrinB2. Interestingly, *grl* is downstream of Notch and interference with Notch signaling resulted in a similar vascular phenotype (Zhong *et al.,* 2000). These results suggest that a Notch-*grl* pathway is important for controlling the assembly of the first embryonic arteries.

Bmx. Bmx is a member of the Tec family of tyrosine kinases, which are expressed predominantly in hematopoietic cells. Bmx is also expressed in the endothelium, specifically in large arteries and the endocardium during both prenatal and postnatal life (Ekman *et al.,* 2000; Rajantie *et al.,* 2001). The gene was not expressed in arterioles, capillaries or veins, with the exception of a weak signal in the superior and inferior vena cavae near the heart (Rajantie *et al.,* 2001). The targeted disruption of Bmx had no effect on vascular development (Rajantie *et al.,* 2001). Available evidence suggests that Bmx is regulated by the Tie-2 and the VEGFR-1 receptors (Rajantie *et al.,* 2001). In contrast to EphrinB2 or Dll4, which are expressed before circulation is established, Bmx was activated between E10.5 and E12.5, suggesting that this signaling molecule is not involved in the early stages of arterial versus venous identity and raising the possibility that its expression is regulated by physiological cues, such as blood flow/pressure.

Other arterial endothelial cell-specific genes. Several other genes have been shown to be expressed predominantly on the arterial side of the circulation during development and/or during adult life, including the transcription factors Sox-13 (Roose *et al.,* 1998), EPAS-1 (Tian *et al.* 1997), and HRTl-3 (Nakagawa *et al.,* 1999), and the transmembrane receptor protein tyrosine phosphatase μ (Bianchi *et al.,* 1999). The degree to which these properties are genetically pre-determined and whether or not they play an active role in mediating arterial fate has not been determined.

3.
Vascular bed-specific VEGF signaling

Several investigations point to the existence of arterial and venous differences in VEGF signaling. For example, in chicken embryos neuropilin-1, which is an isoform-specific VEGF receptor able to bind the heparin-binding VEGF isoforms (Soker *et al.,* 1998), is expressed predominantly on the venous side of the circulation, whereas neuropilin-2, a splice-form specific VEGF receptor, is expressed on the arterial side of the circulation in a pattern that is analogous to that of ephrinB2 (Herzog *et al.,* 2001).

The murine VEGF is alternatively spliced to yield three isoforms, $VEGF_{120}$, $VEGF_{164}$, and $VEGF_{188}$. Each of these isoforms has unique binding properties and functions. In a recent study, mice were genetically engineered to express a single VEGF isoform (Stalmans *et al.,* 2002). Mice that expressed only $VEGF_{164}$ were phenotypically normal, whereas $VEGF_{120}$ expressing mice exhibited severe defects in retinal vascular outgrowth and patterning. In contrast, $VEGF_{188}$ expressing mice displayed normal venular outgrowth but impaired arterial development specifically in the retina, changes that were not observed in the kidney or heart. The authors concluded that the shorter isoforms of VEGF induce expression of arterial markers or that the $VEGF_{188}$ isoform is unable to provide

spatial guidance or differentiation cues for retinal arteriolar endothelial cells (Stalmans *et al.,* 2002).

Other studies have uncovered novel VEGF-like molecules that are specific to certain organ types. For example, an endocrine gland-derived VEGF (termed EGVEGF) was identified as a mitogen specific for the endothelium of steroidogenic glands (LeCouter *et al.,* 2001). Although this protein does not demonstrate structural homology to the VEGF family, it is functionally similar. For example, like VEGF, EG-VEGF is upregulated by hypoxia and induces endothelial cell migration, proliferation and fenestration (LeCouter *et al.,* 2001). In dot blot analyses of tissue RNA arrays, EG-VEGF mRNA was limited to steroidogenic endocrine glands. Most remarkably, the biological effects of EG-VEGF, under both *in vitro* and *in vivo* conditions, were limited to the endothelium from steroidogenic glands, suggesting that the vascular-specific effects of EG-VEGF are determined not only by the availability of the growth factor but also the capacity of the endothelial cell to respond to the signal. Based on these data, it seems likely that other site-specific signaling networks exist and that they contribute in a critical way to the generation and/or maintenance of endothelial cell heterogeneity.

4.
Lymphatic vessels versus systemic vessels

Lymphatic vessels are anatomically and structurally distinct from blood vessels. The endothelial cells lining the lymphatic capillaries have large intercellular gaps. In addition, the small lymphatic vessels typically lack continuous basement membrane and supporting cells, such as pericytes and smooth muscle cells. Lymphatics are present in most organs and are believed to derive from the jugular and perimesonephric regions and perhaps from lymphangioblasts residing in the lateral mesoderm (Karkkainen *et al.,* 2002). However, the mechanisms that underlie lymphatic development are poorly understood. Recent efforts have focused on identifying markers that are unique to this subset of blood vessels. For example, the VEGFR-3 tyrosine kinase receptor (also known as Flt-4) is unique among the VEGF receptors in that it is expressed predominantly in lymphatic endothelial cells. During embryogenesis, VEGFR-3 is expressed as early as E8.5 in the angioblasts of the head mesenchyme, cardinal vein and the allanotois (Kukk *et al.,* 1996). Later during development, expression becomes increasingly restricted to the lymphatic vessels (Kukk *et al.,* 1996). A critical role for VEGFR-3 signaling in lymphangiogenesis was established in studies of patients with familial lymphoedema. This disease is manifested by hypoplasia of lymphatic vessels and secondary swelling of the extremities. Some patients with this disorder have been found to carry a missense mutation of the VEGFR-3 gene (Karkkainen *et al.,* 2000). The over-expression of VEGF-C, the ligand for VEGFR-3, results in hyperplasia of lymphatic vessels in transgenic mice (Jeltsch *et al.,* 1997), adding additional support for the role of VEGFR-3 in lymphatic development.

Other markers that are restricted to lymphatic endothelium include the podocyte cell-surface mucoprotein podoplanin (Breiteneder-Geleff *et al.,* 1997, 1999), the homeobox gene product proxl (Karkkainen *et al.,* 2002; Wigle and Oliver, 1999), the lymphatic vessel endothelial hyaluronan recptor-1 (or LYVE-1) (Banerji *et al.,* 1999), and the beta-

chemokine receptor D6 (Nibbs *et al.,* 2001). The extent to which these or other gene products contribute to the determination of the lymphatic lineage remains to be determined.

5.
Circulating endothelial cells

It has been hypothesized that endothelial cells and hematopoietic cells arise from a common progenitor cell, the hemangioblast (Choi, 2002; Choi *et al.,* 1998). To date, however, the identity of this cell remains elusive (Mikkola and Orkin, 2002). Recent studies suggest that endothelial cell progenitors may persist in adult life. Indeed, there is evidence that endothelial precursors reside in the bone marrow, circulate freely in the blood, maintain the capacity to proliferate, and contribute to the formation of new blood vessels (Asahara *et al.,* 1997, 1999; Kalka *et al.,* 2000; Reyes *et al.,* 2002). These circulating precursor cells should be distinguished from circulating differentiated endothelial cells, which have low proliferative capacity and do not participate in neovascularization (Lin *et al.,* 2000; Peichev *et al.,* 2000). The role that circulating endothelial precursor cells play in physiological or pathophysiological angiogenesis has yet to be established. Moreover, it is not clear whether bone marrow-derived progenitors represent a single clone or whether they carry different genetic programs and information for unique endothelial cell phenotypes.

6.
Disease states

6.1
Primary pulmonary hypertension

Primary pulmonary hypertension (PPH) is a fatal disorder that occurs in familial (FPPH) and sporadic forms. PPH represents a primary defect of the pulmonary arterial vasculature that is characterized by pulmonary artery lesions consisting of medial hypertrophy, concentric laminar intimal fibrosis and plexiform lesions with obstruction of the arterial lumen (Loscalzo, 2001). A central mechanism underlying these lesions is the uncontrolled proliferation of endothelial cells. Many patients with FPPH have defects in a gene that encodes bone morphogenetic protein receptor II *(BMPR2)* a member of the TGF-β superfamily of receptors (Deng *et al.,* 2000; Lane *et al.,* 2000; Machado *et al.,* 2001; Newman *et al.,* 2001). Ligand binding of bone morphogenetic protein with the extracellular domain of bone morphogenetic protein receptor II results in the phosphorylation of bone morphogenetic protein receptor I, ultimately resulting in the activation of the Smad family of transcription factors. Over 25 mutations in *BMPR2* have been identified in families with FPPH. Many of these mutations predict a loss-offunction, suggesting that the normal function of bone morphogenetic protein receptor II in the lung is to inhibit cell growth. It is not clear whether the vascular phenotype is a result of the

mutation in the endothelium or whether it is secondary to a defect in *BMPR2* signaling in vascular smooth muscle cells (Loscalzo, 2001). It is interesting to note that only a small fraction of individuals that carry the *BMPR2* mutations develop pulmonary hypertension. Presumably, the genetic mutation conspires with co-modifiers, including local environmental signals or other mutations, to induce lung-specific proliferative lesions in a subset of carriers.

In contrast to FPPH, the plexiform lesions in sporadic forms of PPH contain monoclonal populations of endothelial cells that exhibit somatic microsatellite site mutations and reduced protein expression of TGF-βRII as well as Bax, a proapoptotic member of the Bcl-2 family (Geraci *et al.,* 2001; Yeager *et al.,* 2001). It has been proposed that these somatic mutations may promote the growth of a rare pulmonary artery phenotype, resulting in the characteristic vascular lesions (Stevens *et al.,* 2001). The molecular mechanisms that underlie the initiation, localization and progression of this disease remain to be defined.

6.2
Hereditary telangiectasia

Hereditary telangiectasia is an autosomal dominant vascular dysplasia that is characterized by abnormalities in endothelial cells, vascular dilations and arteriovenous malformations, particularly in the pulmonary, hepatic and cerebral circulation (Guttmacher *et al.,* 1995). Telangiectasias arise when post-capillary venules dilate and directly fuse with arterioles, thereby bypassing the capillary network (Braverman *et al., 1990*). Hereditary telangiectasia may be caused by loss-offunction mutations in one of two genes, namely endoglin or *ACVRL1* (activinlike receptor kinase I, also known *as ALK1*) (Johnson *et al.,* 1996; McAllister *et al.,* 1994). Endoglin is an accessory protein member of the type III class of TGF-βreceptors, whereas *ACVRL1* encodes a type I receptor for the TGF-β superfamily of growth factor receptors in endothelial cells. The distribution of vascular lesions differs between the two genetic variants of hereditary telangiectasia. For example, pulmonary and cerebral arteriovenous malformations are more common in the patients with mutations in endoglin compared with *ACVRL1* (Berg *et al.,* 1996; Cymerman *et al.,* 2000). Thus, while environmental stimuli, such as shear stress, estrogens, and blood pressure may play a role in determining the location of vascular lesions, the underlying genetic defect is also an important determinant of the clinical phenotype.

Based on genetic mouse models, Oh and his colleagues have proposed a model of disease in which mutations in endoglin or *ACVRL1* result in unimpeded activation of a pro-migratory TGF-β-dependent signaling pathway in capillary endothelial cells (Oh *et al.,* 2000). The net effect is the formation of arteriovenous connections between dilated venules and arterioles with no intervening capillary bed (Oh *et al.,* 2000). Mice that carry a single copy of the endoglin gene display a multi-organ vascular phenotype that is similar to human hereditary telangiectasia (Bourdeau *et al.,* 2001). Interestingly, the severity score and age of onset were reported to vary according to the genetic background (Bourdeau *et al.,* 2001). These findings point to the existence of modifier genes that control angiogenesis and contribute to the phenotypic expression of the disease. Indeed,

the co-inheritance of modifier alleles may account for the heterogeneous clinical manifestations observed between and within families of patients with hereditary telangiectasia.

Other studies of mice that are null for *ACVRL1* have revealed a primary vascular defect shortly after the *de novo* formation of the central vascular tree with abnormal endothelial remodeling (Urness *et al.,* 2000). The phenotype was associated with a loss of ephrinB2 expression from the arterial tree (Urness *et al.,* 2000). These data suggest that *ACVRL1* plays a role in assigning arterial-venous identity following the formation of the primary capillary plexus of the yolk sac and central vascular tree of the embryo proper.

Mutations in the TGF-β superfamily of growth factor receptors have been implicated in the pathogenesis of both hereditary telangiectasia and PPH, suggesting a common link between these two disease states. Indeed, patients with hereditary telangiectasia may develop pulmonary hypertension that is clinically and pathologically indistinguishable from FPPH (Trembath *et al.,* 2001). In a recent study, the molecular analysis of kindreds with combined hereditary telangiectasia and PPH revealed mutations in *ACVRL1* (Trembath *et al.,* 2001). *ACVRL1* is expressed in endothelial cells, and a mutation of the gene has been predicted to interfere with its ability to inhibit the growth-promoting actions of TGF-β signaling (Loscalzo, 2001). It is noteworthy that the *ACVRL1* gene is expressed at higher levels in the rat lung compared with other tissues (Panchenko *et al.,* 1996), providing a possible explanation for the site-specific lesions in PPH.

6.3
Hemangiomas

Juvenile hemangiomas are benign tumors of vascular endothelial cells. They are the most common tumors of infancy, affecting up to 10% of all Caucasian infants. Most hemangiomas appear within the first year of life, occurring as single cutaneous or subcutaneous lesions, predominantly in the head and neck region. Although the majority of hemangiomas are sporadic, a small number may arise as part of an autosomal dominant trait (Blei *et al.,* 1998). The excessive proliferation of endothelial cells in this disorder must arise from a defect that is either extrinsic or intrinsic to the endothelium. Two recent studies provided compelling evidence that endothelial cells derived from hemangiomas are in fact clonal in origin (Boye *et al.,* 2001; Walter *et al.,* 2002). These findings suggest that somatic mutation(s) alter the growth potential of the endothelium, perhaps through gain-in-function of a pro-angiogenic factor or loss-of-function of an antiangiogenic factor. In another study, hemangioma-derived endothelial cells were shown to express higher levels of Tie-2 and to respond more avidly to angiopoietin-1 compared with normal human primary endothelial cells, implicating a role for the Tie-2angiopoietin axis in the pathogenesis of this disorder (Yu *et al.,* 2001). From the standpoint of endothelial cell heterogeneity, it would be interesting to know how environmental or genetic modifiers interact with one another to localize the majority of the vascular lesions to the region of the head and neck.

6.4
Tumor angiogenesis

Without blood vessels, tumors cannot grow beyond a critical size. Tumor vessels develop through the sprouting of pre-existing vessels and perhaps through the seeding of circulating endothelial precursors (Carmeliet and Jain, 2000; Davidoff *et al.,* 2001; Reyes *et al.,* 2002). Tumor vessels are morphologically and functionally distinct (Carmeliet and Jain, 2000). Compared with normal vasculature, for example, tumor vessels are highly disorganized, tortuous and dilated, resulting in chaotic and variable flow patterns. Moreover, they display increased permeability (Hashizume *et al.,* 2000). Tumor endothelial cells express target molecules that are otherwise undetectable in normal endothelium. Using *in vivo* phage display technology, Arap *et al.,* identified markers that are preferentially expressed in tumor endothelium (Arap *et al.,* 1998). In another study, a comparison of gene expression patterns in endothelial cells derived from normal human colonic tissue or from human colorectal cancers revealed a panel of markers that were specifically upregulated in tumor endothelium (St Croix *et al.,* 2000). Some of these genes have also been localized in the vascular endothelium of mouse embryos (Carson-Walter *et al.,* 2001), suggesting that they are markers for neo-angiogenesis.

The endothelium of tumor vessels may acquire different phenotypes, depending on both the type and site of the tumor. For example, a recent study showed that for the same tumor type, the diffusion of large molecules was significantly faster in a cranial window compared with a dorsal chamber (Pluen *et al.,* 2001). In addition, endothelial cells derived from different tumors have been shown to retain distinct phenotypes *in vitro* (Alessandri *et al.,* 1999).

Despite the differences between tumor and normal endothelium, it is important to emphasize that tumor endothelial cells are derived from normal tissue and are therefore genetically stable. The prevailing view is that tumor endothelium, whether derived from surrounding blood vessels or from circulating precursors acquires a phenotype that depends on properties of both the surrounding tissue and the tumor environment. To date, there is no evidence that the differential properties of tumor endothelium are genetically pre-determined.

7.
Summary

It is widely appreciated that endothelial cells are heterogeneous in structure and function. Initial studies designed to address the molecular basis of endothelial cell heterogeneity uncovered a critical role for the microenvironment. More recently, sophisticated molecular approaches have provided insight into the importance of genetic determinants in mediating at least some of the phenotypic differences between endothelial cells.

Indeed, existing data support a dual role for both lineage determination and microenvironmental cues in mediating site-specific phenotypes *(Figure 1)*. The decision to form a vein or an artery is made earlier during embryonic life before the onset of circulation. Whether this fate is pre-programmed in the earliest angioblast or is governed

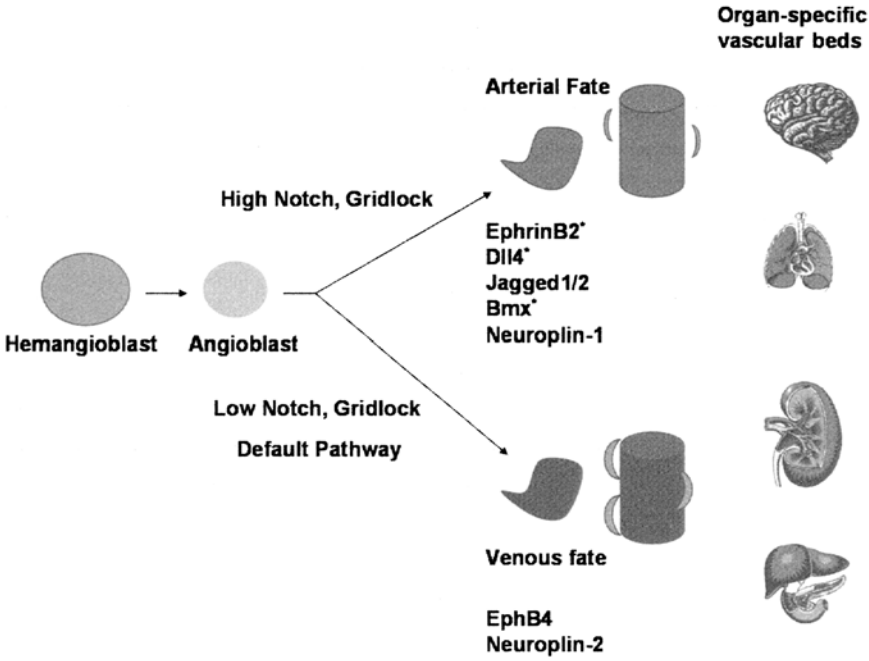

Figure 1. Development of site-specific properties of the endothelium. The putative hemangioblasts differentiate into the angioblasts, which are then fated to form the endothelial lining of either arteries or veins. Several markers are expressed early during this process and may play an important role in determining cell fate or mediating the migration and/or coalescence of similarly fated cells. In some cases (denoted by the *), the arterial-specific markers persist in adulthood. During or following development, the endothelium acquires properties that are uniquely adapted to the underlying tissue (brain, lung, liver, kidney are shown). Although this is particularly true at the level of the microcirculation, phenotypic differences also exist between the large arteries and veins of different organs. In contrast to the role of genetic programs in governing arterial-venous identity, phenotypic heterogeneity at the level of the microcirculation is governed predominantly by cues residing in the extracellular environment.

by subtle, as yet unrecognized, extracellular signals in the embryo proper remains to be determined. Once endothelial cells reach their tissue of residence, their ultimate phenotype is largely dictated by signals in the microenvironment. Finally, in disease states, a combination of genetic and environmental factors is likely to determine the nature and location of the vascular phenotype(s).

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2
Control of vascular morphogenesis by Eph receptor and ephrin signaling

Amelia Compagni and Ralf H.Adams

1. Introduction

Receptor tyrosine kinases (RTKs) are well-established regulators of morphogenic processes during vertebrate embryonic development. Their signaling controls a multitude of fundamental cellular processes including proliferation, differentiation, protection against cell death and guidance of migrating cells or neuronal growth cones (Flanagan and Vanderhaeghen, 1998; Holder *et al.,* 1999; Huang *et al.,* 2001; Schlessinger, 2000). In the cardiovascular system, crucial roles for RTKs binding soluble ligands, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) and angiopoietin, have been firmly established (Gale and Yancopoulos, 1999; Risau and Flamme, 1995). Among other functions, some of these molecules regulate the *de novo* assembly of blood vessels (vasculogenesis) and their branching into complex networks (angiogenesis), processes by which the vascular system is initially formed in embryos and subsequently expanded and adapted to match the increasing requirements of growing tissues (Flamme *et al.,* 1997). More recently, the molecular repertoire controlling vascular growth and organization has been expanded to include the large RTK family of Eph receptors (named for the expression of the founding molecule in an *e*rythropoietin-*p*roducing *h*epatocel-lular carcinoma cell line) and their ligands, called ephrins (for *Eph* receptor *in*ter-acting molecules) (EN Committee, 1997). Unlike soluble growth factors, ephrins are cell surface-attached molecules and require direct cell-cell contact for interaction with Eph receptors. Their most remarkable feature is the unusual ability of ephrin molecules to induce bidirectional signal transduction into both ligandand receptor-expressing cells. The Eph/ephrin system provides a complex and, because of the large number of genes involved, costly signaling tool kit used not only in the vasculature but in a wide range of body structures. The previously described functions of the gene family in the developing nervous system also encourages speculation about

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possible parallels or links between neuronal and vascular network formation (Shima *et al.,* 2000).

Although the roles of Eph/ephrin molecules, as well as of other RTK families, have been mainly studied during development of the embryonic cardiovascular system, there is growing evidence that many mechanistic and molecular aspects are also applicable to blood vessel formation in adults. With the exception of the female reproductive system, only few blood vessels are newly formed in the adult body but the vasculature retains a remarkable growth potential, which becomes evident during wound healing or various pathological conditions, such as diabetic retinopathy, rheumatoid arthritis or tumorigenesis (Folkman, 1995). First observations indeed seem to indicate an involvement of Ephs and ephrins in the process of neoplastic progression and tumor vascularization (see below), but, as long as fundamental functional aspects of these molecules are poorly understood, it will prove difficult to estimate their relevance as potential therapeutic targets.

This chapter will summarize what is known about signaling by Eph/ephrin molecules and their biological role in the cardiovascular system. Furthermore, we have included several examples illustrating their function in other organs and particularly in the nervous system in which Eph receptors and ephrins have been studied for a much longer time and are therefore better understood.

2.
The Eph receptor family

2.1
Structure and functional domains of Eph receptors

The Eph receptors comprise the largest family of receptor tyrosine kinases including 14 distinct members in higher vertebrates. Eph receptors and ligands have also been described in invertebrates such as the fruitfly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans* (George *et al.,* 1998; Scully *et al.,* 1999). Two subclasses (A and B) can be distinguished according to their binding affinity and sequence homology. EphA receptors (EphA1 to 8) bind and are activated by A-ephrins (ephrin-A1 to 6), which are tethered to the cell surface by a glycosylphosphatidylinositol (GPI) modification. In contrast, EphB receptors (EphB1 to 6) bind to B-class ligands (ephrin-B1 to 3), which are class I transmembrane proteins with short cytoplasmic domains. As shown in *Figure 1,* receptor-ligand interactions are highly promiscuous but restricted to members within A or B subclasses, respectively. As exceptions, ephrin-B2 and, with higher affinity, ephrin-B3 are also able to bind the EphA4 receptor.

The extracellular domain of Eph receptors consists of an aminoterminal globular domain followed by a cysteine-rich region and two fibronectin type III domains. Several studies (Himanen *et al.,* 1998; Labrador *et al.,* 1997) have shown that ephrin-binding is mediated by the globular domain and, for the example of EphB2, its crystal structure both in absence and presence of ligand interaction has been published (Himanen *et al.,* 1998,

2001). These results led to the assumption that binding is normally initiated in trans between molecules presented on adjacent cells, with a first heterodimerization step involving one ligand and one receptor molecule. Subsequently, higher order complexes are formed in which two ligands interact with two receptors and this in turn triggers downstream signaling events (Himanen *et al.,* 2001). There is prevailing opinion that ephrins can be presented in different clustering states, ranging from monomers to higher order complexes such as tetramers. Although it has so far proved impossible to measure the extent of multimerization of membrane-bound Ephs, ephrins or their binding complexes, one study showed that different clustering states of recombinant soluble ephrin-B1 can induce alternative Eph receptor signaling (Stein *et al.,* 1998b). Whereas binding of either soluble ephrin-B1 dimers or tetramers are sufficient to trigger EphB1 tyrosine phosphorylation, only the latter induces recruitment of low-molecular weight phosphatase (LMW-PTP) to the receptor and measurable cell attachment responses (Stein *et al.,* 1998b). Soluble fusion proteins consisting of the ligand extracellular domain and the human immunoglobulin G constant region (Fc), which are presumably dimeric, are commonly used by many laboratories. In order to elicit responses in most cell culture and biochemical assays, the Fc fusion proteins require further multimerization with anti-Fc antibodies (Adams *et al.,* 1999; Bennett *et al.,* 1995; Elowe *et al.,* 2001; Stein *et al.,* 1998b). However, this common practice does not provide evidence of similar requirements for the membrane-anchored form of native ephrins. Moreover, the mechanisms by which different clustering states of the ligands might be established on the cell surface are unknown, despite the recent description of a dimerization motif in the ephrin-B2 extracellular domain (Toth *et al.,* 2001).

Following ligand binding, activation and signaling of Eph receptors seem to depend on the phosphorylation of two cytoplasmic tyrosine residues, which are part of a well-conserved 10 amino acid motif in the intracellular juxtamembrane region *(Figure 2). In vitro* these tyrosines are major substrates for the receptor autokinase activity (Binns *et al.,* 2000; Kalo and Pasquale, 1999) and their mutation almost completely abolishes ligand-induced tyrosine phosphorylation as exemplified for EphB2 (Binns *et al.,* 2000) and EphA4 (Kullander *et al.,* 2001). Recent studies have provided the structural basis for the Eph receptor autoinhibition by the unphosphorylated juxtamembrane region (Wybenga-Groot *et al.,* 2001).

Additional tyrosine residues become phosphorylated upon ligand binding within the kinase domain and in the C-terminal sterile alpha motif (SAM), the two main structural domains found in the cytoplasmic part of Eph receptors *(Figure 2).* The role of the SAM domain during receptor signaling remains elusive—its deletion does not impair EphA4 function *in vivo* (Kullander *et al.* 2001)—but structural studies have revealed a potential role in receptor dimerization (Stapleton *et al.,* 1999; Thanos *et al.,* 1999). At their very carboxyterminus, most Eph receptors also present a conserved VXV binding motif for PDZ domaincontaining proteins (named after the founding molecules PSD95, Discs-large and ZO-1) and indeed interaction with the molecules AF-6 (Hock *et al.,* 1998), GRIP, PICK1 (Torres *et al.,* 1998) and the recently discovered transmembrane protein ARMS (Kong *et al.,* 2001) has been demonstrated. PDZ proteins have been implicated in numerous

cellular processes including protein complex assembly, localization and clustering of transmembrane proteins and signaling, all of which might be relevant for Eph receptors.

It is noteworthy, that two Eph receptors, namely EphBS, which has a truncated cytoplasmic region, and EphB6, which contains a point mutation in its kinase domain, lack kinase activity. It has been initially suggested that these molecules might function as molecular reservoirs modulating the ligand availability for signaling-competent Eph receptors. Such speculations have not been confirmed since EphBS remains an orphan receptor without any known ligand interactions. In the case of EphB6, binding of ephrin-B2 (see *Figure 1*) has been recently reported (Munthe *et al., 2000*). Despite the lack of

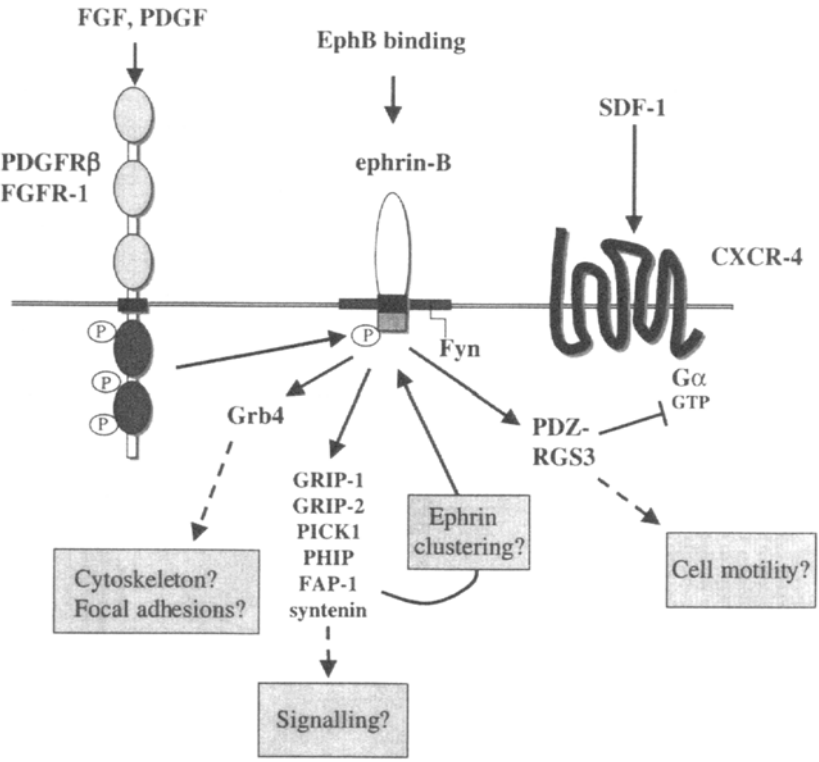

Figure 1. Binding between Eph receptors and ephrins. Very strong (K_D< 1 nM; black squares), robust (K_D=1–10 nM; dark grey), weaker interactions (K_D> 10 nM; light grey) and lack of binding (-) are indicated. Qualitative results without dissociation constants are shown as 'b'. Conflicting reports exist in some cases (grey boxes containing minus symbol). A detailed summary of binding properties and dissociation constants can be found in Flanagan and Vanderhaeghen (1998). Results for EphB6 and ephrin-A6 are from Munthe *et al.,* (2000) and Menzel *et al.,* (2001). Amino acid residue homologies among A-and B-class molecules were calculated using PileUp of the GCG Sequence Analysis Software Package Version 3.2. Distance along the axis was shortened for display reasons and is not directly proportional to difference between sequences.

kinase activity, cross-linking of EphB6 with a monoclonal antibody directed against its extracellular domain, which presumably mimics multimerization by ligand binding, leads to an unexpected increase of tyrosine phosphorylation levels in cultured cells. The cytoplasmic adapter molecule CB1 and the Src homology 2 domain-containing tyrosine phosphatase-1 (SHP-1) were implicated in this process suggesting that EphB6, similar to other members of the gene family, can trigger signaling events (Luo *et al.,* 2001).

2.2
Cellular responses to Eph receptor signaling

Eph receptor activation results in conformational changes and tyrosine phosphorylation of docking sites, which permits recruitment of a multitude of SH2 domain proteins including the Ras GTPase-activating protein p120rasGAP (Hock *et al.,* 1998), the cytoplasmic tyrosine kinases Src (Zisch *et al.,* 1998) and Fyn (Ellis *et al.,* 1996; Hock *et al.,* 1998), AB1 and Arg (Yu *et al.,* 2001), the adapter proteins Nck (Stein *et al.,* 1998a), Crk (Hock *et al.,* 1998), Grb2, GrblO (Stein *et al.,* 1996), the src-like adapter protein SLAP (Pandey *et al.,* 1995a), the R-Ras binding protein SHEP-1 (Dodelet et al., 1999),thep85 subunit of PI3-kinase(Pandey et al., 1994) and the low-molecular-weight phosphatase (LMW-PTP) (Stein *et al.,* 1998b) *(Figure 2).* Unlike growth factor receptors, Eph family kinases are inefficient in stimulating cell proliferation in fibroblasts or epithelial cells and are involved rather in the control of axon guidance, neural crest cell migration and endothelial cell sprouting (see following paragraphs). In all these processes, remodeling of the cytoskeleton, cell adhesion and interaction with the extracellular matrix have to be precisely regulated. Several studies have begun to link signaling events downstream of activated Eph receptors to specific biological responses. For example, stimulation of EphB2 by ephrin-B1 in neuronal cells leads to growth cone collapse and neurite retraction. Concomitantly, the protein p62dok is tyrosine phosphorylated and forms a complex with p120rasGAP, Nck and Nik (Nck-interacting kinase) (Becker *et al.,* 2000; Holland *et al.,* 1997). Nck has been shown to bind proteins such as PAK and WASP that are directly involved in actin polymerization, and both p120rasGAP and Nck might regulate the activity of Rho/Rac/cdc42 GTPases, critical players in the control of cell motility. Interaction with Nik, which can activate JNK kinases, appears necessary for ephrinBl-induced cell attachment to fibrinogen, presumably by modulation of integrin activity (Becker *et al.,* 2000; Stein *et al.,* 1998a). Stimulation of EphB2 by ephrin-B1 in the same cellular system also leads to downregulation of the RasMAPK pathway via p120rasGAP and thus contributes to cytoskeletal reorganization (Elowe *et al.,* 2001).

Besides Nck/Nik, interaction of Eph receptors with low-molecular-weight phosphatase also appears to be required for the modulation of cell attachment via integrins. Stimulation by ephrin-B1 multimers promotes endothelial cell attachment and assembly in capillary-like structures, processes that are dependent on the recruitment of LWM-PTP and the engagement of integrins avß3 and a5ß1 (Huynh-Do *et al.,* 1999). Although LWM-PTP is known to regulate p190Rho-GAP, the precise molecular link between its function and integrin activation is unknown.

More recently, a member of the guanine exchange nucleotide factor (GEFs) for RhoGTPases, named ephexin, has been shown to interact with EphA receptors. Ephexin is constitutively bound to Eph receptors and upon ligand-binding induces RhoA activation, Cdc42 and Racl inhibition with consequent cell morphology changes (Shamah *et al.,* 2001). At this point, ephexin represents the most direct link between Eph receptors and the actin cytoskeleton.

The multidomain protein AF-6, which also contains a single PDZ domain, provides another potential link between Eph receptor signaling and the cytoskeleton. AF-6 binding to activated Eph receptors appears to be very important for the proper localization and clustering of these molecules to areas of cell-cell contact (Buchert *et al.,* 1999), presumably by interaction with tight junction proteins such as ZO-1 and JAM (Ebnet *et al.,* 2000). Moreover, AF-6 is known to bind profilin, a regulator of actin polymerization (Boettner *et al.,* 2000).

It is interesting to note that Eph receptor signaling does not always increase cellular attachment. For example, activation of EphB2 artificially expressed in fibroblasts leads to R-Ras phosphorylation, presumably via SHEP-1 activity, and loss of cell adhesion (Zou *et al.,* 1999). Similarly, stimulation of EphA2 by ephrinA-1 suppresses integrin-mediated adhesion and cell spreading. Within minutes after ligand binding, the tyrosine phosphatase SHP-2 is recruited to EphA2, followed by dephosphorylation of the focal adhesion kinase (FAK), paxillin and dissociation of an EphA2/FAK complex (Miao *et al.,* 2000). Frisen and colleagues provided a possible explanation for the puzzling ability of Eph receptors to increase or reduce cellular adhesion (Holmberg *et al.,* 2000). The authors observed that, although ephrin-A5 is involved in cell repulsion, ephrin-A5 null mutants exhibit neural tube defects consistent with a disrupted cell adhesion-promoting function. They then discovered expression of three different splice variants for the ephrin-A5-binding receptor EphA7 on neural folds, a full length one and two truncated forms lacking the kinase domain. Co-expression of these variants *in vitro* shifted the cellular response from repulsion to adhesion (Holmberg *et al.,* 2000). However, it is not clear whether this is a general mechanism used by other Eph receptors and the *in vivo* role of individual splice variants remains to be addressed.

There is some evidence in the literature suggesting a connection between Eph receptors and adhesion molecules of the cadherin family. EphA2 is localized in cell-cell contact areas in normal breast epithelia, but in breast adenocarcinoma cells lacking E-cadherin the receptor is diffusely distributed and enriched within membrane ruffles (Zantek *et al.,* 1999). Inactivation of the E-cadherin gene in embryonic stem (ES) cells not only diminishes adherens junctions and cell-cell contacts but also changes the cellular localization and expression levels of EphA2 (Orsulic *et al.,* 2000). In addition, injection of EphA4 mRNA in early *Xenopus laevis* embryos causes cell dissociation at the blastomere stage and loss of adherens junctions both of which can be rescued by overexpression of C-cadherin (Winning *et al.,* 1996,2001).

2.3
Cross-talk of Eph receptors with other signaling pathways

It is now widely accepted that a complex network of interdependent molecular interactions rather than isolated pathways mediates cellular signaling. Eph receptors and RTKs of other gene families do share many downstream effector molecules and several examples of signaling cross-talk have been described *(Figure 2)*. For example, activation of the EphB2 receptor leads to downregulation of the ERKl/2 MAPK pathway suggesting that the Eph/ephrin system might modulate the cellular response to signaling by other receptors (Elowe *et al.,* 2001). This ability of Eph receptors is not unique to the B-class since stimulation of EphA receptors by ephrin-Al also inhibits ERKl/2 activation induced by soluble growth factors such as EGF, PDGF and VEGF in epithelial or endothelial cells (Miao *et al.,* 2001). Thus Eph receptor signaling might inhibit mitogenic responses triggered by other RTKs.

A poorly understood link exists between Eph receptors and the unusual orphan receptor Ryk (related to tyrosine *k*inases), which lacks kinase activity (Halford *et al.,* 2001; Katso *et al.,* 1999). Ryk has been shown to associate specifically with EphB2, EphBS and EphA7 and, like Eph receptors, Ryk can bind the PDZ domain protein AF-6. Tyrosine-phosphorylation of Ryk is induced by activation of B-class Eph receptors and, remarkably, Ryk-deficient mice exhibit craniofacial defects reminiscent of EphB mutant animals (Halford *et al.,* 2000).

The navigation of growing nerve fibers to their correct targets was one of the first processes for which a role of Eph receptors and ephrins was revealed and therefore it is not surprising that much of our knowledge comes from studies in the developing nervous system (Flanagan and Vanderhaeghen, 1998; Klein, 2001; Wilkinson, 2001). More recent evidence demonstrates that Eph/ephrin molecules retain important functions in the adult brain, after axons have reached their cellular targets and established neuronal connections. For instance, EphB2 has been shown to tyrosine phosphorylate the transmembrane heparan sulfate proteoglycan syndecan-2, which leads to syndecan-2 clustering and dendritic spine formation. This process can be blocked by expressing dominant negative EphB2 receptor in cultured hippocampal neurons (Ethell *et al.,* 2001). Although the exact role of syndecans is not well understood, there is increasing evidence that they are involved in signaling and interact with cytoskeletal proteins such as syntenin (Grootjans *et al.,* 1997), synectin (Gao *et al.,* 2000), synbindin (Ethell *et al.,* 2000) and the membrane-associated calcium/calmodulin-dependent serine protein kinase CASK (Hsueh *et al.,* 1998).

Another link exists between Eph RTKs and the NMDA-type glutamate receptor (NMDAR), a key player in the control of activity-dependent synaptic plasticity and memory formation in the brain (Drescher, 2000). In neuronal cells, ephrin-B binding to EphB2 induces a direct interaction with NMDAR (Dalva *et al.,* 2000) and NMDAR-mediated functions are reduced in EphB2 null mice (Grunwald *et al.,* 2001; Henderson *et al.,* 2001). The modulation of NMDAR activity might be independent of EphB kinase activity since a truncated EphB2 receptor, which lacks the kinase domain but still interacts with NMDA receptors via the extracellular region, can rescue the synaptic defect observed in

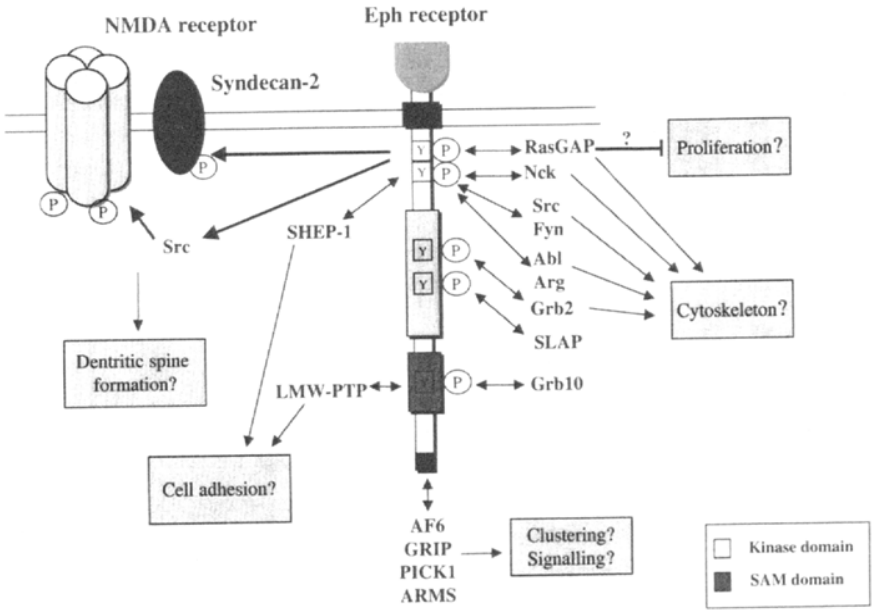

Figure 2. Signaling by Eph receptors. Transmembrane area, juxtamembrane region (black box), kinase domain (light grey), SAM domain (dark grey), C-terminal PDZ-binding motif (black) and tyrosine phosphorylation sites (Y) in the cytoplasmic part of Eph receptors are shown. Molecular interactions, signaling pathways and cellular responses based on observations in different cell types are summarized.

EphB2 null mice (Grunwald *et al.,* 2001). However, ephrin-B2-induced calcium influx via the NMDAR in cultured primary cortical neurones requires EphB kinase activity (Takasu *et al.,* 2002). Less controversial is the finding that Eph signaling results in activation of Src family kinases, which form complexes with NMDA receptors, and NMDAR tyrosine phosphorylation (Grunwald *et al.,* 2001; Takasu *et al.,* 2002). Therefore, cross-talk with the NMDA-receptor pathway rather than direct Eph signaling appears to be a critical determinant in this process.

Interestingly, a new transmembrane protein, ARMS, characterized by a SAM and a PDZ-binding motif, has been shown to associate both with Eph receptors and Trk neurotrophin receptors (Kong *et al.,* 2001). Hence, it is possible that Eph receptors might be involved in the modulation of additional receptor signaling pathways.

3.
The ephrin ligands

3.1
'Forward' and 'reverse' signaling

Work over recent years has provided compelling evidence that ephrin molecules can mediate signal transduction in two fundamentally different ways. As ligands, they can bind and activate their receptors triggering specific responses in the receptor-expressing cell. This process, which is frequently referred to as 'forward' signaling, appears to be very similar to the well-studied activation of RTKs by growth factor ligands. But unlike these soluble molecules, both A-and B-class ephrins are also capable of signaling in an additional, exceptional fashion and elicit downstream responses into the cell presenting them on its surface. The term 'reverse' signaling is now commonly used to describe such events, which, together with 'forward' Eph receptor activation, lead to bidirectional signaling by Eph and ephrin molecules.

3.2
Ephrin-A reverse signaling

Genetic experiments in the nematode *C. elegans* provided a good example for Eph/ephrin signaling. The *C. elegans* genome contains genes encoding one Eph receptor, called vab-1, and at least four different ephrins, efn-1 to 4. All these ligands are GPI-anchored and thus referred to as A-class molecules albeit they share extensive sequence homology with the extracellular domains of vertebrate B-ephrins (Wang *et al.,* 1999). Inactivation of the vab-1 gene results in multiple defects including the incomplete closure of epidermal sheets forming the body wall of the *C. elegans* larvae, a phenotype which is mirrored by mutants lacking several efn genes. Conversely, loss of the Vab-1 kinase activity due to a mutation in the kinase domain gives rise to a much milder phenotype indicating different roles for kinase-dependent, i.e. 'forward signaling', and kinase-independent Eph receptor signaling (Chin-Sang *et al.,* 1999; Wang *et al.,* 1999). The latter might include 'reverse' signaling by the A-class ephrins as well as other yet unknown mechanisms of signal transduction.

In vertebrates, ephrin-A ligands have important functions in the developing nervous system, e.g. during axonal navigation, by providing repulsive guidance cues for nerve fibers expressing Eph RTKs (Knöll and Drescher, 2002; O'Leary and Wilkinson, 1999). But A-class ligands, such as ephrin-A5, are also found on neuronal cells and it was shown that the interaction with recombinant soluble EphAS can induce outgrowth of neuronal processes (Davy *et al.,* 1999). This is remarkably different from the repulsive responses caused by Eph receptor activation in similar assays and raises the question as to how A-ephrins can signal in the absence of a cytoplasmic domain. As GPI-anchored molecules, A-class ligands are localized to sphingo-lipid/cholesterol-enriched raft membrane microdomains (Davy *et al.,* 1999). Upon activation by EphA receptor binding, ephrin-A5 becomes associated with the Src family kinase Fyn. The kinase can attach to the

cytoplasmic side of the lipid rafts in the plasma membrane and it was shown that its catalytic activity is indeed required for ephrin-mediated responses (Davy *et al.,* 1999). Concomitantly, Rho and Rho kinase become activated (Wahl *et al.,* 2000) and increased cell adhesion is mediated by the activation of β1 integrin (Davy and Robbins, 2000). A newly identified 120-kDa protein, that becomes tyrosine phosphorylated specifically after ephrin-A stimulation, might represent the link between ephrin ligands and integrins (Huai and Drescher, 2001).

Recent work has also provided some insight into a mechanism that terminates Eph/ephrin-mediated cell-cell communication. Ephrins of both subclasses contain a conserved 10 amino acid motif located roughly in the middle of the extracellular part. A similar sequence motif is also present in some non-related molecules such as the Notch-ligand Delta, tumor necrosis factor-α and in the amyloid precursor protein (APP) implicated in Alzheimer's disease (Hattori et al., 2000). John Flanagan and his co-workers were the first to demonstrate that this sequence represents a recognition site for the metalloprotease Kuzbanian/ADAM10 in ephrins. They could show that ephrin-A2, after engagement with EphA receptor, is cleaved in its extracellular juxtamembrane region and released from the cell surface (Hattori *et al.,* 2000). Mutation of the metalloprotease recognition motif delays a typical receptor-mediated response, the withdrawal of EphA-expressing neuronal growth cones from ephrin-A2-presenting cells (Hattori *et al.,* 2000).

3.3
Reverse signaling by ephrin-B proteins

Class B ephrin ligands are characterized by a high degree of conservation of their carboxyterminal tails. A sequence stretch covering the last 33 amino acids is identical in ephrin-B1 and ephrin-B2 and only slightly varied in ephrin-B3, and key motifs such as five tyrosine residues and a PDZ-binding motif are perfectly conserved. Upon binding to Eph receptors, ephrin-B proteins are phosphorylated by an unknown tyrosine kinase (Brückner *et al.,* 1997). Although *in vitro* Src is able to phosphorylate the ephrin-B cytoplasmic domain (Brückner *et al.,* 1997), there is currently no evidence for a physiological significance of this process. It has been shown by mass spectrometry that the same tyrosine residues of ephrin-B1 are phosphorylated *in vitro* and in the embryonic retina (Kalo *et al.,* 2001). Interestingly, the major *in vivo* phosporylation site in ephrin-B1 is Tyr331, in position-3 from the C-terminus and part of the PDZ domain-binding motif. Several proteins can associate with this site such as the adapters GRIP1, GRIP2, syntenin and PDZ-RGS3, the protein kinase C-interacting protein Pick1, the phosphotyrosine phosphatase Fap-1 and PHIP, which is related to PAR-3, a regulator of polarity in *C. elegans (Figure 3)* (Brückner *et al.,* 1999; Lin *et al.,* 1999; Torres *et al.,* 1998). Interaction with these PDZ proteins might facilitate the localization of ephrin-B1 into sphingo-lipid/cholesterol-enriched raft membrane microdomains, control multimerization and contribute to downstream signaling events (Brücknerer al., 1999). Most of the interacting PDZ proteins are apparently bound to ephrin-B ligands in the absence of stimulation and, so far, tyrosine phosphorylation of ephrins does seem to not alter this interaction (Lin *et al.,* 1999).

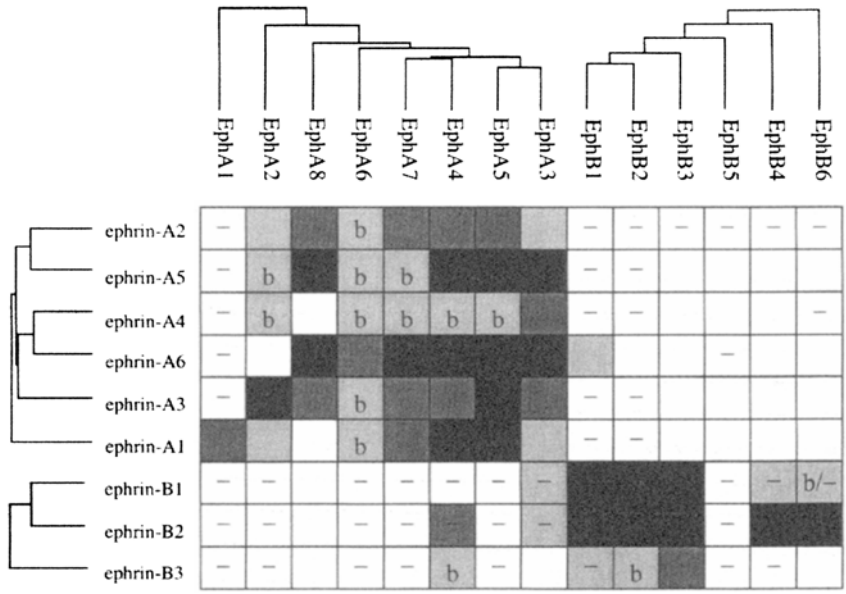

Figure 3. Ephrin-B signaling and molecular cross-talk. Signaling by B-class ephrins through phosphotyrosine-binding and PDZ domain proteins and cross-talk to growth factor receptors and the chemokine receptor CXCR-4 are shown.

Several different approaches have convincingly proved that ephrin-B ligands are indeed receptor-like signaling molecules. Biochemical studies have shown that the SH2-adapter protein Grb4 (also known as Nck-2) can bind a tyrosine-phosphorylated stretch of 22 amino acid residues in the ephrin-B1 cytoplasmic region (Cowan and Henkemeyer, 2001). Association with Grb4 appears to be required for ephrinmediated responses such as increase of FAK catalytic activity, redistribution of paxillin, loss of focal adhesions and disassembly of F-actin stress fibers. The Grb-4 SH3 domain can in turn bind a number of other proteins (PAKl kinase, axin, Abi-1, CAP dynamin), many of which are involved in cytoskeletal regulation. Also, Cbl-associated protein CAP can be co-immunoprecipitated with activated ephrinB1 (Cowan *et al.* 2001). Ephrin ligands are therefore able to recruit signaling machinery that mediates responses in the ligand-expressing cells.

What is known about the biological role of B-class ephrin 'reverse' signaling? Eph-ephrin interaction can mediate contact-dependent repulsion between adjacent cell populations artificially expressing receptors and ligands, respectively (see *Figure 5*). This process requires bidirectional signaling since EphB2 or ephrin-B2 molecules lacking their cytoplasmic domains (?C) fail to restrict cell intermingling in the same assay. Remarkably, reconstructed bidirectional signaling combining unidirectional 'forward' and 'reverse' signals elicited by ephrin-B2?C and EphB2?C, respectively, restores cellular repulsion (Mellitzer *et al.,* 1999). In genetic experiments in mice, loss of the receptor kinase activity results in milder nervous system defects than the full inactivation of the gene, as shown

for EphB2 and EphA4 (Henkemeyer *et al.,* 1996; Kullander *et al.,* 2001). In a complementary approach, replacement of the endogenous ephrinB2 with the carboxyterminally truncated ephrin-B2?C causes midgestation lethality similar to ephrin-B2 null mutants. While ephrin-B2?C mice display the vascular defects observed in the ephrin-B2 knock-out mice, more subtle abnormalities in the development of the branchial arches are rescued (Adams *et al.,* 2001). These results have shown that the cytoplasmic domain of ephrin-B2 and reverse signaling are not required for guidance of Eph receptor-expressing cranial neural crest cells, which presumably involves 'forward' signaling, but are necessary for mouse vascular morphogenesis.

Some reports have highlighted the capacity of ephrin-B ligands to cross-talk with other receptor-mediated signaling pathways. Stimulation of NIH 3T3 cells with PDGF leads to tyrosine phosphorylation of ephrin-B1 independently from Ephreceptor binding (see *Figure 3*). The cytoplasmic domain of ephrin-B1, in turn, can suppress formation of transformed cell foci induced by a number of activated tyrosine kinases including PDGFRß (Bruckner *et al.,* 1997). Similarly, activated FGF receptor triggers ephrin-B1 phosphorylation presumably by direct interaction of the intracellular domains. Expression of ephrin-B1 in *Xenopus* blastomeres leads to loss of cellular adhesion and dissociation of the embryos, which can be blocked by FGF-induced tyrosine phosphorylation of the ligand. Since ephrin-B1 requires the C-terminal PDZ-binding motif to trigger dissociation of blastomeres, it appears possible that FGFR-induced phosphorylation might interfere with the recruitment of PDZ domain proteins (Chong *et al.,* 2000; Jones *et al.,* 1998).

As a final example, the interaction between ephrin-B1 and PDZ-RGS3, a cytoplasmic protein containing a PDZ and a regulator of heterotrimeric G-protein signaling domain, selectively inhibits activity of the chemokine SDF-1, the ligand for the G-protein coupled receptor CXCR4 (Lu *et al.,* 2001). The mechanism by which ephrins regulate PDZ-RGS3 activity is unclear but mere recruitment of the RGS protein to the membrane appears not to be sufficient (Lu *et al.,* 2001).

In conclusion, the current picture is that the cytoplasmic domain of transmembrane ephrins can signal in at least two separate but possibly interconnected ways: by the recruitment of adapter proteins to phosphotyrosine residues and PDZ protein binding. The localization in membrane rafts and the activation of raftassociated Src family kinases might provide a third mechanism.

4. Eph/ephrins in the cardiovascular system

During embryogenesis, two distinct processes lead to the formation of new blood vessels: vasculogenesis and angiogenesis. Vasculogenesis involves the *de novo* differentiation of endothelial cells from mesodermal precursors or angioblasts (Risau and Flamme, 1995) and gives rise to structures such as the dorsal aorta, the cardinal vein, the heart endocardium and the primary capillary plexus in the yolk sac. Later, new blood vessels arise from the pre-existing vasculature by sprouting, proliferation and migration of endothelial cells in a process called angiogenesis or vascular remodeling (Risau, 1997). In

the brain, new blood vessels are also generated by intussusception, i.e. the splitting of blood vessels by the insertion of tissue pillars (Patan, 2000).

4.1
A-class Eph receptors and ephrins

Ephrin-A1 was originally cloned as B61, an immediate early response gene induced by tumor necrosis factor-α (TNF-α) in human umbilical vein endothelial cells (HUVECs), several years before it was identified as an Eph receptor ligand (Bartley *et al.,* 1994; Beckmann *et al.,* 1994; Holzman *et al.,* 1990; Shao *et al.,* 1994). Later work provided further evidence for roles of ephrin-A1 in the vascular system. It is a chemoattractant for endothelial cells and has angiogenic activity *in vitro* as well as in cornea pocket assays *in vivo* (Daniel *et al.,* 1996; Pandey *et al.,* 1995b). Consistent with an angiogenic role, ephrin-A1 mRNA is expressed at relevant sites including dorsal aortas, primary head veins, intersomitic vessels and limb bud vasculature during morphogenesis of the cardiovascular system in mouse embryos (Flenniken *et al.,* 1996; McBride and Ruiz, 1998). The receptor EphA2 seems to be an important interactor of ephrin-A1 in endothelial cells: the receptor is activated in response to stimulation with ephrin-A1 and a dominant negative form of EphA2 inhibits formation of capillary-like tubes by HUVECs (Myers *et al.,* 2000; Ogawa *et al.,* 2000; Pandey *et al.,* 1995b). Furthermore, ephrin-A1 and EphA2 proteins were detected on the vasculature of both surgically removed human and experimentally grown mouse tumors (Ogawa *et al.,* 2000). Although the findings described above strongly indicate that ephrin-Al and EphA2 can control angiogenesis, no overt vascular defects have been observed in EphA2-deficient mice (Chen *et al.,* 1996).

4.2
The role ofEphB receptors and ephrinB ligands in vascular development

Several EphB receptors and ephrin-B ligands are expressed in the embryonic cardiovascular system but only recent findings have implicated these molecules in the control of blood vessel formation. Anderson and colleagues (Wang *et al.,* 1998) discovered by insertion of a LacZ cassette in the ephrin-B2 gene that the ligand is expressed on arterial endothelial cells from the earliest stages of angiogenesis in the mouse embryo (see *Figure 4*). Similar findings in zebrafish and chick make the ligand a useful marker for arteries, despite its expression in many structures outside the cardiovascular system. In contrast, the cognate receptor EphB4 is found predominantly on veins, although its expression is not completely excluded from arteries (Adams *et al.,* 1999; Gerety *et al.,* 1999; Wang *et al.,* 1998), suggesting that the EphB4-ephrin-B2 interaction might help to define arterial-venous (AV) identity (see below). The targeted inactivation of the ephrin-B2 gene in mice results in severe cardiovascular defects, growth retardation and embryonic lethality by embryonic day 11 (E11). Mutant blood vessels in heads and yolk sacs fail to undergo angiogenic remodeling and remain highly reminiscent of the primitive capillary plexus, the uniform honeycomb-shaped network of blood vessels

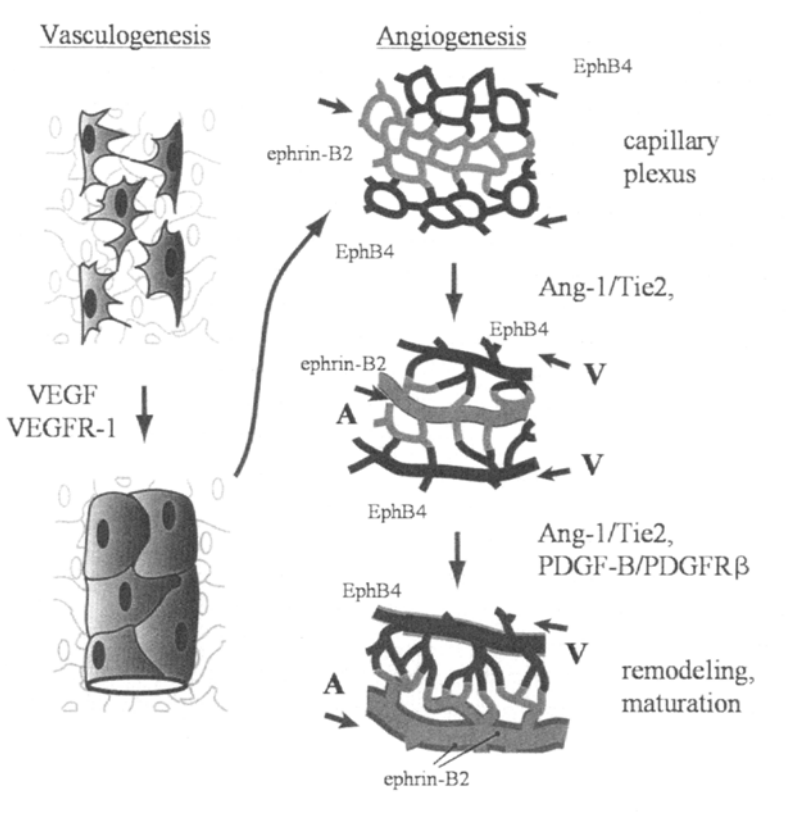

Figure 4. Formation of new blood vessels by vasculogenesis and angiogenesis. Direct assembly of blood vessels by endothelial precursors (vasculogenesis, left) and angiogenic remodeling of a primitive primary plexus into a hierarchical vascular network (right) are schematized. Arterial (grey) and venous (black) domains expressing ephrin-B2 and EphB4 and the direction of blood flow (small arrows) are shown. During angiogenesis, a relatively uniform primary plexus is progressively remodeled into a hierarchical network of large and small blood vessels. As part of the maturation program, smooth muscle cells (dark grey) are recruited to larger vessels predominantly on the arterial side and express ephrin-B2. Some key molecules in the processes of vasculogenesis (VEGF/VEGFR-1), angiogenesis (Ang-l/Tie2) and smooth muscle cell recruitment (PDGF-B/PDFGRβ are indicated.

formed by vasculogenesis (see *Figure 4*) (Adams *et al.,* 1999; Wang *et al.,* 1998). The anterior cardinal vein and, with a low penetrance, the dorsal aorta, which are both formed directly by the assembly of angioblasts, are also disrupted indicating that the ligand might be required for some aspects of vasculogenesis. Mutant hearts, which normally express both ephrin-B2 and EphB4 on their endocardium, are enlarged, incompletely looped and display reduced myocardial trabeculation (Adams *et al.,* 1999; Wang *et al.,* 1998). In conclusion, the loss of ephrin-B2 produces cell autonomous as well as non-autonomous

defects by affecting both arterial and venous beds and possibly their interaction. Disruption of the EphB4 gene essentially mimics the ephrinB2 mutant phenotype, indicating that interaction between these molecules and perhaps bidirectional signaling are required for fundamental steps during vascular morphogenesis (Gerety *et al.,* 1999). The generation of mice expressing a C-terminally truncated form of ephrin-B2 by a 'knock-in' approach results in similar cardiovascular defects, indicating essential roles of the ligand cytoplasmic domain (Adams *et al.,* 2001).

Besides ephrin-B2, the highly related ephrin-B1 is also expressed in endothelial cells but seems not to be restricted to the arterial or venous domains (Adams *et al.,* 1999; Bruckner *et al.,* 1999). Although its role *in vivo* is still unclear, ephrin-B1, similar to ephrin-B2, is capable of exerting angiogenic activity *in vitro*. Stimulation of the endogenous EphB1 receptor on human renal microvascular endothelial cells (HRMECs) by ephrin-B1 induces capillary-like structures (Daniel *et al.,* 1996; Stein *et al.,* 1998b). Moreover, treatment of adrenal-cortex microvascular endothelial cells (ACE) with recombinant ephrin-B1 or ephrin-B2 protein leads to sprout formation in a three-dimensional fibrin gel, similar to responses elicited by VEGF or angiopoietin-1 (Adams *et al.,* 1999). The third B-class ligand, ephrin-B3, is expressed in the embryonic heart (Gale *et al.,* 1996) but mice deficient for this gene do not present any cardiovascular defects. The receptor EphB3, able to bind all three B-class ephrins, is also expressed on the endothelium of veins (cardinal veins, venous intersomitic vessels) and some arteries such as the aortic arches (Adams *et al.,* 1999; Ciossek *et al.,* 1995). EphB2 is expressed in mesenchymal cells adjacent to certain vessels, suggesting that its interaction with endothelial ligands might regulate the communication between the endothelium and surrounding tissues. Although neither EphB2 or EphBS are required for cardiovascular morphogenesis during embryonic development, mutants lacking both receptors displayed vascular defects and embryonic lethality with a partial penetrance of 30% (Adams *et al.,* 1999).

4.3
Eph/ephrin-mediated interactions between endothelial cells and other tissues

Eph receptors and ephrins are expressed in many structures and cell types of vertebrate embryos. Besides controlling morphogenesis of various organ systems, Eph/ephrin interactions might also help to co-ordinate different developmental processes. One such example is somites, segmented blocks of paraxial mesoderm located on both sides of the neural tube, which will later develop into a variety of tissues such as bone, muscle and skin. Multiple Eph/ephrin gene family members are expressed on somites in spatially restricted patterns before any differentiation and specialization of the mesodermal tissue becomes visible. Ephrin-B1, for example, labels the prospective dermomyotome in the dorsal part of somites in mouse (Wang and Anderson, 1997). The receptors EphB2 and EphA4 are expressed in rostral somite halves whereas ephrin-B2 is restricted to caudal halves, which will later form sclerotome and various skeletal structures (Durbin *et al.,* 1998,2000; Krull *et al.,* 1997; Wang and Anderson, 1997). The precise role of these molecules in somites is not well understood but disruption of normal Eph-ephrin signaling

by overexpressing ephrin-B2 or dominant negative EphA4 in zebrafish embryos results in abnormal somite shapes and boundaries (Durbin *et al.,* 1998). Motor neuron axons, which grow from the spinal cord to their peripheral targets, and neural crest cells, which delaminate from the dorsal neural tube to migrate in ventral direction, are patterned by somite-derived cues and avoid posterior/caudal somite halves. Although several different signals are presumably involved in this process, both motor neurons and neural crest cells express Eph receptors and show repulsion from ephrin-B ligands *in vitro* (Krull *et al.,* 1997; Wang and Anderson, 1997). Intersomitic blood vessels (ISV) grow from cardinal veins and dorsal aortas, which are located laterally and ventrally from the spinal cord, along the intersomitic spaces into the dorsal embryo and initially avoid sprouting into the somitic tissue itself. At least some intersomitic blood vessels express EphB3 and EphB4 and, consistent with loss of a repulsive cue, ISVs in ephrin-B2 deficient mice frequently branch into the somitic mesenchyme (Adams *et al.,* 1999). Likewise, disruption of EphB4 signaling by a dominant negative EphB4 receptor or ectopic expression of ephrin-B2 in Xenopus embryos allowed veins to sprout abnormally into the somitic tissue (Helbling *et al.,* 2000).

Additional evidence for involvement of ephrin-B2 in the communication between endothelial and mesenchymal cells is provided by observations in null mutant embryos: mesenchymal cells or pericytes surrounding blood vessels are abnormally rounded (Wang *et al.,* 1998) and expression levels of the RTK Tie2 and its ligand angiopoietin-1, which have been previously implicated in EC-support cell interactions (Gale and Yancopoulos, 1999), are reduced (Adams *et al.,* 2001).

Further complexity was added by the finding that ephrin-B2 expression, besides marking arterial endothelium in embryonic and adult mice, expands progressively during development to the surrounding pericytes and vascular smooth muscle cells (SMC) (Gale *et al.,* 2001; Shin *et al.,* 2001). Stromal OP-9 cells artificially expressing ephrinB2 are able to promote not only proliferation and sprouting of ephrin-B2 expressing endothelial cells, but also recruitment and proliferation of SMCs *in vitro* (Zhang *et al.,* 2001). In accordance with these results, activation of Eph-ephrin signaling can induce proliferation of cultured SMCs and expression of the receptor EphB2 is upregulated by arterial denudation when vascular repair mechanisms are activated (Woods *et al.,* 2002). The role of the Eph/ephrin system in SMCs and pericytes requires further studies, but it seems that receptor-ligand interactions might involve all cellular components of the cardiovascular system suggesting roles beyond vasculogenesis and angiogenesis.

Eph/ephrin molecules might also control tissue morphogenesis and communication between different cell types in the developing murine kidney. Ephrin-B2 expression, besides labeling glomerular endothelial cells, is also found on podocyte progenitors and mesangial cells whereas EphB4 is restricted to venous endothelial cells (Takahaski *et al.,* 2001). The receptor EphB1, which can also interact with ephrin-B2, is found on smooth muscle cells, glomeruli and possibly mesangial cells in newborn and adult kidneys. The expression of ephrin-B1 in kidney on arterioles and glomeruli (Daniel *et al.,* 1996) completes a complex picture with multiple possible ligand-receptor interactions during glomerular microvascular assembly.

4.4
Arterial-venous identity

The restriction of ephrin-B2 and EphB4 expression to arteries and veins, respectively, initially raised the question whether or not these molecules could determine the AV identity of blood vessels during embryonic development. Recent work has identified additional molecules that are differentially expressed only on the arterial endothelium and has begun to unravel the mechanisms underlying the AV decision. The important role of the bHLH transcription factor gridlock was identified by a large-scale chemical mutagenesis screen in zebrafish (Zhong *et al.,* 2000). Gridlock is expressed in cells that originate in the lateral posterior mesoderm and converge at the midline to form the primordium of the dorsal aorta. In mutants lacking the transcription factor or in animals in which expression was experimentally reduced, arterial regions are progressively lost, a phenotype that is most apparent at the anterior bifurcation of the aorta, and EphB4-expressing veins are expanded (Zhong *et al.,* 2000, 2001). Hence, gridlock might determine and maintain the arterial identity by activating a specific program or by repressing factors critical for the venous fate. Gridlock is downstream of Notch signaling and several members of this receptor family are expressed on endothelial cells in zebrafish and mouse. In addition, a new member of the Delta family of Notch ligands, Dll-4, is selectively expressed in arterial endothelium (Shutter *et al.,* 2000). Mice lacking Notch1 show vascular defects with disruption at the anterior bifurcation of the aorta (Krebs *et al.,* 2000) and in zebrafish Notch deficiency leads to loss of arterial markers, such as ephrin-B2 (Lawson *et al.,* 2001). From these results, it appears that Notch and gridlock are so far the earliest markers of arterial differentiation and the Eph/ephrin system is regulated, at least on the mRNA level, by this pathway.

Two recent publications made use of the chick-quail graft system to determine if endothelial cells have rigid AV commitments or if some degree of plasticity allows them to change their arterial or venous fate. Endothelial cells coming from sufficiently young quail donor embryos can colonize all blood vessels in their chick hosts but, consistent with plasticity, they express ephrin-B2 only upon incorpo ration in arteries but not veins (Moyon *et al.,* 2001; Othman-Hassan *et al.,* 2001). In contrast, grafted cells from older quail donors show progressively less plasticity and populate selectively arteries or veins indicating stringent AV commitments in more mature vascular beds (Moyon *et al.,* 2001). These results also show that arterial and venous endothelial cells do not intermingle within blood vessels, a mechanism which might help to establish an AV boundary by restricting EC migration (*Figure 5*).

A similar restriction of cell movement is found in the embryonic hindbrain where the neural tube is segmented into morphological units called rhombomeres. Eph receptors are expressed in rhombomeres r3 and r5 whereas ligands, such as ephrin-B2, predominate in even-numbered segments (r2, r4, r6) suggesting that these molecules interact at the boundaries between the segments and help to restrict cell migration (see *Figure 5*) (Klein, 1999). When Eph receptors or ephrin-B2 are artificially misexpressed within even-or odd-numbered zebrafish rhombomeres or these cells migrate to the segment boundaries thus minimizing their contact with the surrounding cells. Conversely, no such sorting was

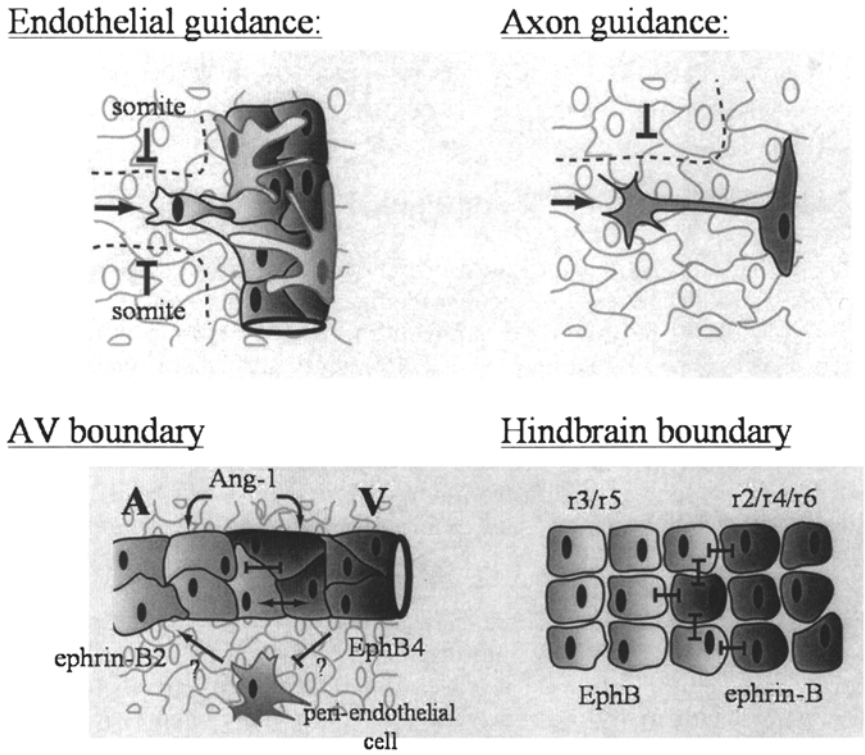

Figure 5. Analogous cellular responses in the nervous and vascular systems. Top: Sprouting of intersomitic vessels is patterned by somite-derived repulsive cues and similar signals are used for axonal navigation. Chemoattractants (arrows) provide the driving force for endothelial sprouting or axon outgrowth. Bottom: Restriction of cell movement by boundaries at the AV interface and between hindbrain rhombomeres. Cells misexpressing EphB receptor or B-ephrins preferentially move to the rhombomere boundary to minimize Eph-ephrin interactions. Binding between ephrin-B2 and EphB4 at the interface between arteries and veins may restrict intermingling of endothelial cells and help to create an AV boundary. The same interaction may also promote angiogenic sprouting. Expression of ephrin-B2 and EphB4 away from the AV boundary may help to control recruitment of peri-endothelial cells. Ang-1/Tie2 and ephrin/Eph molecules have both been implicated in endothelial-mesenchymal interactions.

observed for cells overexpressing Eph RTKs in r3/r5 or ephrin-B2 in r2/r4/r6 (Xu *et al.*, 1999). It is tempting to speculate that Eph/ephrin signaling might restrict intermingling of endothelial cells between arterial and venous vascular beds just as it restrains migration of neural cells in hindbrain rhombomeres.

In the light of all the studies described above, EphB4 and, in particular, ephrin-B2 have become established markers for veins and arteries in a range of vertebrate species. They seem to act downstream of an initial AV decision made by Notch and Delta molecules. The specific roles of Eph/ephrin molecules on arteries or veins remain to be determined.

5. Roles for Eph receptors and ephrins in cancer?

Although critical functions for Eph/ephrin molecules in vascular morphogenesis have been established, our current knowledge makes it difficult to assess their potential roles during tumor development and their suitability as therapeutic targets. However, accumulating evidence indicates that Eph/ephrin molecules are frequently overexpressed in tumors or cancer cell lines. For example, the founding member of the gene family, EphA1, was isolated and named from an erythropoietin-producing hepatoma cell line and high levels of expression have been shown in breast, lung and colon carcinomas (Maru *et al.,* 1988). NIH 3T3 fibroblasts overexpressing EphAl are able to form foci in soft agar and tumors in nude mice (Maru *et al.,* 1990). Expression of EphA2, which can bind a wide range of A-class ligands including ephrin-A1, is increased in nearly all melanoma cell lines while it is normally not expressed in melanocytes (Easty *et al.,* 1995). EphA2 levels are significantly higher in cell lines derived from distant metastases than from primary melanomas. Ephrin-Al levels also increase with progressive melanoma development, with up to 70% of analyzed human metastatic tumors being positive for ephrin-A1 (Easty *et al.,* 1999). Aggressive melanoma cells that are able to form tubular networks in three-dimensional cultures, referred to as 'vasculogenic mimicry', display high levels of EphA2 phosphorylation (Hess *et al.,* 2001). Moreover, analysis of human breast cancer cell or Kaposi's sarcoma cell xenografts in mice showed that both tumor and endothelial cells express EphA2 and ephrin-A1, with EphA2 being tyrosine phosphorylated and activated (Ogawa *et al.* 2000).

The B-class receptor EphB2 is overexpressed in one third of 31 human tumor cell lines examined, in 75% of gastric tumors and in some esophageal and colon cancers (Kiyokawa *et al.,* 1994). High levels of EphB4 were found in human breast carcinoma cell lines and in breast carcinomas, especially at very invasive stages (Berclas *et al.,* 1996).

In mouse mammary tumors induced by the oncogene H-Ras, high levels of EphB4 expression correlate with invasiveness and degree of malignancy. In normal mammary glands, EphB4 is predominantly localized in the myoepithelial cells surrounding the ducts and alveoli whereas ephrin-B2 is found on luminal epithelial cells. Remarkably, expression of the ligand is lost and progressively replaced by EphB4 in tumors. Artificial deregulation of EphB4 expression in transgenic animals delays the normal development of mammary epithelium but does not result in a higher incidence of mammary tumors. However, in transgenic mice expressing both EphB4 and neuT genes in the mammary gland, tumorigenesis is accelerated and formation of metastasis in the lung is enhanced compared to neuT animals (Munarini *et al.,* 2002; Nikolova *et al.,* 1998). This phenotype might be due to the altered tissue architecture and increased invasiveness in EphB4-overexpressing mammary glands.

Two independent groups have inserted a LacZ cassette in the murine ephrin-B2 gene and showed that blood vessels at sites of neovascularization, e.g. in the corneal micropocket assay, wound healing or tumors, are ephrin-B2-positive (Gale *et al.,* 2001; Shin *et al.,* 2001). In tumors formed by Lewis lung carcinoma or B16 melanoma cells, the majority, but not all, of the blood vessels express ephrin-B2. Hence, in tumors,

endothelial cells are seemingly induced to sprout from preexisting ephrin-B2-expressing vessels, presumably arteries, and not venous capillaries as commonly believed (Gale *et al.,* 2001; Shin *et al.,* 2001).

Further work is required to gain insight into the role of the Eph/ephrin system in the adult organism under normal and pathological conditions. However, given their prominent roles during vascular morphogenesis in the embryo, it seems well worth investigating if ephrin-B2 and EphB4 could be putative therapeutic targets for the tumor vasculature. Other Eph receptors and ephrins may have important functions on mature blood vessels although they are not indispensable for vascular morphogenesis in the embryo. Several reports have shown that Eph/ephrin molecules are sometimes expressed on cancer cells, which might affect the recruitment of blood vessels but also tissue invasion and formation of metastasis. It will be important to understand how stimulation or disruption of Eph-ephrin signaling might influence these processes. But before we consider therapeutic applications, many fundamental aspects of Eph/ephrin biology such as regulation of their expression, function in the endothelium and other cell types, signaling cross-talk and the role of specific receptor-ligand interactions need to be studied in much greater detail. Considering the complexity of the Eph/ephrin system, this task might take more than a few years and include further surprises.

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3
The angiopoietins

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The angiopoietins, angiopoietin-1–4, represent an unusual family of high-affinity ligands. The angiopoietins, signaling through the tyrosine kinase Tie receptor system, comprise a unique agonisf.antagonist relationship that mediates a number of angiogenesis-related, endothelial cell activities. Early evidence indicated that angiopoietin 1 stimulated the Tie signaling cascade while angiopoietin 2 functioned to inhibit this activity. Additional evidence indicates that these ligands integrate, through a complex coordination, with other angiogenesisrelated molecules to affect blood vessel formation and function. This chapter reviews current knowledge of the angiopoietins and presents an intricate system of molecular interactions contributing to new blood vessel formation.

1.
Angiopoietin-1

1.1
Isolation and structure of angiopoietin-1

Angiopoietin-1 was originally isolated using secretion-trap expression cloning of the SHEPl-1 human neuroepithelioma cell line and the C2C12ras mouse myoblast cell line (Davis *et al.,* 1996). These cell lines produced a ligand that bound to Tie-2, resulting in Tie2 phosphorylation. cDNA expression libraries from these cell lines were then constructed, and transfected into COS cells. After fixation and permeabilization, cell populations were screened with a molecular probe containing the ectodomain of Tie2 fused to the Fc portion of human IgG1 (Tie2/Fc), and individual cells with Tie2 ligand "trapped" within vesicles were identified. cDNA encoding the ligand was then directly rescued from these cells. The DNA sequences were determined, revealing open reading frames encoding 498 amino acids and sharing 97.6% identity between human and mouse sequences (Davis *et al.,* 1996).

Ang-1 is a glycoprotein of~55 kDa, with several glycosylation sites (Davis *et al.,* 1996). Hydrophobic sequences are located at the N-terminal regions of Ang-1, typical of secretory signal sequences. Weak homology to myosin is found in residues 100–280, with a coiled-coil quaternary structure. Residues 280–498 are quite similar to the fibrinogen protein family. Several isoforms of Ang-1 have been identified. In addition to the full

length mRNA (1.5 kb), there are three alternately spliced species of Ang1 mRNA (Ang-1. 3 kb, Ang-0.9 kb, and Ang-0.7 kb) so far identified. These splice variants were discovered in the megakaryocyte cell line CHRF, and subsequently confirmed in human tumor cell lines, fibroblasts and platelets (Huang *et al.,* 2000). Protein translated from the 0.9-kb isoform, which has a truncated coil-coiled domain, is capable of binding Tie2 but does not activate the receptor. In contrast, the 1.3 kb variant produces a protein that contains an intact coiled-coil domain, but a modified fibrinogen-like domain. The protein derived from this isoform fails to bind Tie2 and is capable of forming a heteromer with the full-length protein (derived from the 1.5 kb transcript). Little is known about the activities of the 0.7 kb transcript, although the protein formed from this variant contains deleted regions in both the coiled-coil and the fibrinogen-like domains. These splice variants may serve as dominant negative molecules for the intact Ang1 (derived from the 1.5 kb transcript), which suggests a greater complexity to the angiogenic process.

Intact Ang-1 binds specifically to Tie-2, with an estimated binding affinity of K_D=3.7 nM based on Scatchard analysis of immobilized ligand (Davis *et al.,* 1996). The coiled-coil region allows for assembly of the protein into multimeric structures, and secreted Ang-1 exists in disulfide-linked oligomeric complexes (Davis *et al.,* 1996; Procopio *et al.,* 1999). Although soluble Ang-1 is capable of forming homo-trimers, it is predominantly linked in higher order homooligomeric complexes (Procopio *et al,* 1999). The Ang-1 coiled-coil domain is 63% identical and 91% similar to Ang-2, although functional hetero-oligomers have not been identified. When the coiled-coil domain of Ang-1 was fused to the fibrinogen-like domain of Ang-2, the oligomerization pattern was consistent with Ang-1, but this heteromer was unable to phosphorylate Tie2 (Procopio *et al.,* 1999). The homo-oligomeric structure of Ang1 suggests that it activates Tie2 by bridging receptor molecules, although bridging alone appears to be insufficient to activate endothelial Tie2 (Procopio *et al.,* 1999). The fibrinogen-like domain of Ang-1 is responsible for receptor binding, but this domain appears to bind Tie2 only when dimerized or oligomerized.

In addition to being secreted, Ang1 is incorporated into the extracellular matrix (ECM) of tumor cells. Endothelial cell adhesion causes the release of ECM-associated Ang1, with subsequent Tie2 phosphorylation (Xu and Yu, 2001). The domain within Ang-1 responsible for ECM incorporation appears to be the linker peptide region between the coiled-coil and fibrinogen-like domains. Ang-2 is not incorporated into ECM, and lacks this linker peptide domain (Xu and Yu, 2001). This suggests a local microenvironment regulation of Ang1 by changing its ECM association status, similar to other growth factors such as TGF-ß (Massague *et al.,* 1990).

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1.2
Expression of angiopoietin-1

Early in development (E9-E11), Ang-1 is observed predominantly in the heart (Suri *et al.,* 1996). Using *in situ* hybridization in parallel sections, Ang-1 expression was localized most prominently to the heart myocardium surrounding the endocardium (Suri *et al.,* 1996). Later in development, Ang-1 becomes more widely distributed, most often within the mesenchyme surrounding developing vessels, and in close association with endothelial cells (Davis *et al.,* 1996). In adult tissues, Ang-1 is found in vascular smooth muscle cells and ablumenal cells tightly surrounding vessels, but not endothelial cells (Davis *et al.,* 1996).

1.3
Cardiac development

The critical need for Ang-1 in vascular development was determined through the generation of Ang-1-deficient mice by Suri *et al.* (1996) The Ang-1 deficiency is embryonic lethal at E12.5 and leads to angiogenesis defects similar to Tie-2-deficient mice (Sato *et al.,* 1995) though less severe. The most prominent defects noted in the mouse involved the developing heart where the endocardium is immature and much less intricately folded than in wild-type mice. Furthermore, the mutant hearts are essentially devoid of trabeculae and the endocardial lining appears collapsed and retracted from the myocardial wall as seen in Tie-2-deflcient mice. These data suggest that Ang-1 produced by the myocardium is essential to development of endocardium and trabecular formation.

1.4
Microvascular development and vessel integrity

The role of angiopoietin-1 in vascular development does not invoke "classic" angiogenesis pathways such as endothelial cell proliferation and migration. Unlike VEGF, Ang-1 does not induce proliferation of endothelial cells at concentrations higher than necessary to induce receptor (Tie-2) phosphorylation (Davis *et al.,* 1996). The role of Ang-1 in vasculogenesis appears to be regulating aspects of endothelial cell maturation after the initial stages of proliferation and tubule formation (Suri *et al.,* 1996). The Ang-1-deficient mice were similar to control mice at E11.5 regarding extensive branching of vessels in head region, but the Ang-1-deflcient mice showed a much simpler organizational network, with less distinction between large and small vessels, and fewer and straighter branches. Ang-1 may also influence vascular development through the regulation of Tie-2 expression. In Ang-1 knockouts, Tie-2 levels were substantially reduced, suggesting either that Ang-1 regulates expression of Tie-2, or that endothelial cell levels of Tie-2 depend on the organizational state of the developing vasculature (Suri *et al.,* 1996). Furthermore, the normal expression of Tie-2 after VEGFR expression is consistent with the observation that Ang-1 has a later function in angiogenesis that is distinct from VEGF.

The overexpression of Ang-1 in the skin of transgenic mice provides further evidence for the involvement of Ang-1 in vessel maturation. Transgenic mice demonstrate an increased vascularization in the skin, with more numerous vessels of larger diameter (Suri *et al.,* 1998). Dermal capillaries and venules were more numerous, with uniform increases in size. Quantitative analysis showed a prominent increase in vessel branching. Endothelial cells demonstrated normal cell-cell contact and association with pericytes and fibroblasts. Endothelial cell width was increased. No evidence of plasma leakage, edema, or erythrocyte extravasation was noted, demonstrating the formation of vessels that are mature and well stabilized (Suri *et al.,* 1998).

Ang-1 protects adult vasculature from leakage, countering VEGF and inflammatory agents (Hayes *et al.,* 2000; Thurston *et al.,* 2000). Using an adenoviral gene delivery method, transient expression of Ang-1 was induced in the liver. Animals targeted with ad-VEGF die from widespread edema, but ad-Ang-1 mice appear normal. Thurston *et al.* measured vascular permeability by Evans blue dye via IV injection. Ad-Ang1 mice were treated with mustard oil, a robust inflammatory agent. The Ang-1 mice were resistant to vascular leakage. Ang-1 mice were also resistant to challenge with VEGF. There was no change in vessel morphology in the 5–10 day experimental window, or to 25 days with nude mice. Transgenic animals overexpressing Ang-1 also demonstrated that Ang-1 blocks the permeability effect of serotonin and platelet activating factor (Thurston *et al.,* 1999). Interestingly, Ang-1 did not enhance tumor growth *in vivo,* but rather retarded growth 3-fold. This is consistent with Ang-l's role in vascular stabilization. Ang1 appears to stabilize EC. The increased vascularity associated with Ang-1 may therefore be from decreased pruning and regression that normally accompanies angiogenesis. In one series, only 3/21 breast cancers expressed Ang-1 (Hayes *et al.,* 2000).

Exogenous Ang-1 or Ang-2 alone cannot induce an angiogenic response in *in vivo* angiogenesis assays (Asahara *et al.,* 1998). However, the combination of Ang-1 and Ang-2 with VEGF can alter the angiogenic response observed with VEGF treatment alone. Ang-1 supplementation of VEGF increases perfusion by enhancing capillary density and luminal diameter of the basal limbus artery. The increase in diameter of limbus artery may be compensatory for increased perfusion and augmented flow. However, Ang-2 supplemented VEGF treatment produces longer vessels with isolated sprouting cells at the capillary tip, and luminal diameter is not increased. Treatment with Tie-2/Fc blocks these effects. Collectively, the studies presented in this section suggest that Ang-1 has a profound role in microvascular development, primarily in microvessel remodeling and specialization.

1.5
Recruitment of pericytes

Ang-1-deficient mice demonstrate a role for Ang-1 in the recruitment of periendothelial cells as well. In these mice, the endothelial cells are poorly associated with the underlying matrix, and do not properly recruit peri-endothelial cells (Suri *et al.,* 1996). At tissue folds or branching points, which typically require periendothelial cell recruitment and matrix organization, peri-endothelial cells are absent, endothelial cells appear less flattened, and

collagen-like fibers are scattered. Elsewhere, vessels also show a decreased number of peri-endothelial cells that are separated from rounded EC.

1.6
Matrix interaction

Ang-1 is involved in regulating endothelial cell-matrix interactions and can serve as a substrate for cell adhesion. Carlson *et al.* demonstrated that human umbilical vein endothelial cells (HUVEC) plated onto Ang-1-coated surfaces adhere, spread and migrate (Carlson *et al.,* 2001). This feature appears to be specific to Ang-1 as Ang-2 demonstrates poor cell adhesion and no spreading in similar experiments. Cell adhesion to Ang-1 is not dependent on Tie-2 and not limited to endothelial cells as fibroblasts transfected with or without Tie-2 also adhere and migrate on Ang-1-coated surfaces. This binding and migration appears to be integrin dependent, and can be blocked through treatment with RGD peptides. Furthermore, cells lacking the integrin a5 cannot adhere to Ang-1, but do adhere to Ang-2, and a5 transfection rescues adhesion to Ang-1. Cells rely more heavily on $\beta 1$ to bind Ang-1, but $\alpha v \beta 5$ to bind Ang-2. Additional studies have shown that Ang-1 induces Tek/Tie-2 receptor mediated adhesion and migration of HUVECs *in vitro* (Carlson *et al.,* 2001). Interestingly, this process is dependent upon the alpha integrin subunit and these data suggest a complex interaction of multiple receptors inducing cell adhesion and mobility.

1.7
Cell survival

In vitro evidence suggests that Ang-1 stabilizes endothelial cells and improves vessel element survival (Papapetropoulos *et al.,* 1999). In a collagen overlay angiogenesis assay, HUVECs reorganize into networks (tubes and cords) that "mature" by 12 h, and regress by 24 h without the addition of growth factors. Supplementation of cultures with Ang-1 induced a concentration-dependent increase in the survival of the networks up to 48 h, but does not induce tube formation. This effect can be neutralized by the addition of soluble Tie-2 receptor fused to the Fc portion of immunoglobulin (Tie2/Fc), which sequesters Ang-1. A more robust effect on network survival is seen when VEGF is added in conjunction with Ang-1, suggesting that Ang-1 modulates the VEGF response (Papapetropoulos *et al.,* 1999). The mechanism is not clear. For example, nitric oxide, which is suspected to mediate the angiogenic effects of VEGF, is not released by Ang-1 as tested by cGMP accumulation in HUVEC (Papapetropoulos *et al.,* 1999).

Papapetropoulos *et al.* (1999) also demonstrated that Ang-1 may protect the endothelial cell from apoptotic induction. The removal of growth factors from an endothelial cell culture results in a subdiploid population of cells, and an associated decrease of cells in the G1 phase of the cell cycle. With Ang-1 supplementation during growth factor withdrawal, fewer subdiploid cells (47% to 34%) were identified, and more cells are found in G1 (Papapetropoulos *et al.,* 1999). Hayes *et al.,* (1999) demonstrated similar results for Ang-1 promotion of cell survival mediated by Tie2 engagement, and

potentiated by VEGF and aFGF. Antisense sequences against the start codon for Tie-2 transfected into bovine endothelial cells or 3T3 cells resulted in a dose-dependent loss of Tie-2 protein expression. *In situ,* end-labeling for fragmented DNA showed that cells transfected with the antisense sequence exhibited an 8% apoptosis rate vs 1% with sense transfection and induced a nearly complete cellular detachment.

The Ang-1 protective effect on endothelial cell survival appears to be mediated through an integrin-independent PI3 kinase/Akt pathway, involving a known anti-apoptotic gene, survivin (Kim *et al.,* 2000b); Papapetropoulos *et al.,* 1999). In endothelial cells, Ang-1 increases Akt phosphorylation at Ser473 and Thr308 that can be suppressed by wortmannin, Tie2/fc, and Ang-2. Suppression of Akt phosphorylation by Ang-1 indicates a role for PI3-kinase in this process. Ang-1 treatment of cells has been demonstrated to increase survivin mRNA and protein by a 3–7-fold increase in transcription with no change seen in bcl-2 (also a known anti-apoptotic gene) concentrations. This effect is mediated through Ang-1induced phosphorylation of Akt via tie2, which upregulates survivin transcription. Use of dominant negative survivin negates the Ang-1 protection from apoptosis (Papapetropoulos *et al.,* 1999).

1.8
Sprouting and branching in vitro *and* in vivo

Ang-1 is a strong inducer of endothelial cell sprouting (Kim *et al.,* 2000a; Koblizek *et al.,* 1998; Witzenbickler *et al.,* 1998). Ang-1 induced sprouting in 3-D fibrin gels using a pulmonary arterial endothelial cells (PPAEC) model. VEGF application in this model was substantially stronger. However, Ang-1 induced both non-directional and directional migration of the endothelial cells. The Tie2/Fc reagent blocked this effect.

Directional migration of endothelial cell appears to be mediated through Tie2. Ang1 induces tyrosine phosphorylation of p125(FAK), which is dependent on PI3 kinase. p125 (FAK) plays a key role in regulating dynamic changes in actin cytoskeleton during migration and adhesion. Ang-1 also induced secretion of plasmin and MMP-2, which is inhibited by PI3 kinase inhibitors (Kim *et al.,* 2000a). Endothelial cell release of proteinases is necessary to degrade matrix for endothelial cell migration. Ang-1 suppressed TIMP2 through a PI3 kinase-independent pathway. Paxillin cytoskeleton protein is involved in actin-membrane attachment at sites of cell adhesion and is also associated with p125FAK. Phosphorylation of p125fak produces simultaneous phosphorylation of paxillin. Ang-1 induced phosphorylation of FAK and paxillin within 5 minutes in these cells, while wortmannin or LY294002 blocked this phosphorylation and migration. Alpha2 anti-plasmin suppressed 53% of sprouting activity in this model. The combination of TIMP1 and-2 also suppressed sprouting by 36%. Wortmannin or LY294002 suppressed Ang-1 induced sprouting by 68 or 61% (Kim *et al.,* 2000a). These data suggest that Ang-1-induced migratory effect might be mediated through tyrosine phosphorylation of p125fak, requiring PI3 kinase activity, which may, in turn, enhance cytoskeleton reorganization and secretion of proteinases.

1.9
Endothelial cell chemotaxis

Both Ang 1 and Ang 2 appear to mediate chemotactic responses in cells that express Tek/Tie-2. Witzenbichler *et al.,* (1998), demonstrated that endothelial cells exhibit chemotactic migration towards angiopoietin-1 and that this directed migration was inhibited in a dose-dependent manner by angiopoietin-2. Although Ang-1 acts as a migratory stimulus using a Boyden chamber model, its effect is very weak compared to VEGF (Witzenbichler *et al.,* 1998). Ang-1 is chemotactic, but not chemokinetic for HUVEC and fibroblasts transfected with Tie2. Fibroblasts transfected to express Tie-2 were also attracted to Ang-2, while Ang-2 was not chemotactic for endothelial cells. No proliferative effect on endothelial cells was seen (Witzenbichler *et al.,* 1998). Tie2/Fc blocked endothelial cell chemotaxis, as did 8–10-fold excess Ang-2.

1.10
Recruitment of hematopoietic stem cells (HSC) and circulating endothelial precursor cells (CEP)

Ang-1 induces delayed mobilization of HSC and CEP, while VEGF (delivered by adenoviral produced VEGF) induced rapid mobilization of HSC and CEP expressing VEGFR2 (Hattori *et al.,* 2001). A robust mobilization was seen with sustained elevation of VEGF and Ang-1 by adenoviral vector injection. Combined elevations of both Ang-1 and VEGF lead to induction of hematopoiesis and increased marrow cellularity, followed by proliferation of capillaries and expansion of sinusoidal space (Hattori *et al.,* 2001). Chronic regional expression of VEGF and Ang-1 may regulate hematopoiesis by promoting the extramedullary mobilization and recruitment of HSCs and CEP Both were required to induce significant remodeling of bone marrow vascular architecture, with concomitant mobilization to the extramedullary organs, resulting in splenomegaly. Ang-1 alone did not affect the spleen size. This effect may be related to alteration of adhesion molecule profile, or sustained survival of Tie2 expressing HSC and CEP Ang-1 and VEGF appeared to be critical for mobilization and recruitment of HSC and CEPs, and may play a role in pathogenesis of postnatal hematopoietic dysfunction and splenomegaly.

1.11
Vascular polarity

Ang-1 and Tiel in combination appear to be critical to establish vascular polarity during angiogenesis (Lougha and Sato, 2001). Polarity is one of the fundamental features of pattern formation and is the basis for left-right symmetry. The vasculature is highly heterogeneous with arterial and venous systems that are structurally and functionally non-uniform. Mouse embryos lacking both Ang-1 and Tiel exhibited specific disruption of the right system of the sinus venosus (Lougha and Sato, 2001). At E8.5, Ang1 expression was seen bilaterally. However, at E9.5, expression becomes polarized, although no phenotypic abnormality was detected. Subsequently, the lumens of the cardinal veins

became discontinuous and fragmented on the right side only, while the left system remained unchanged. Differential regulation of the right system and the left system by Ang-1/Tiel precedes establishment of a morphologically discernible asymmetric venous system. Because no abnormality was seen in Ang-1 or Tiel or Tie2 knockout mice, cooperative interaction is suspected in this mechanism.

2.
Angiopoietin-2

2.1
Genetics and structure

Angiopoietin-2 (Ang2) was first discovered by Maisonpierre *et al.,* in 1997 by the use of homology screening to identify a peptide similar to angiopoietin-1 (Ang1) in its ability to bind the tyrosine-kinase-specific receptor Tie2. They determined that this protein is 496 amino acids in length with a signal peptide, an aminoterminal coiled-coil domain, and a carboxy-terminal fibrinogen-like domain. Human Ang2 is ~85% homologous to murine Ang2, and both of these are ~60% identical to their Ang1 counterparts. Grosios *et al.,* (1999) have localized the gene encoding Ang2 to the human chromosome band 8p23.1 using *in situ* hybridization and radiation hybrid mapping techniques.

In chicken, alternative splicing gives rise to three different species of Ang2 mRNAs: Ang2A, Ang 2B, and Ang2C (Mezquita *et al.,* 2000). These three isoforms are also present in humans and codify for proteins with an identical fibrinogenlike carboxy-terminal domain but a different coiled-coil amino-terminal domain. Furthermore, these isoforms have different patterns of expression with Ang2A and especially Ang2C being expressed in immature testis and regressed adult testis undergoing vascular remodeling. Conversely, Ang2B is only detectable in adult testis at low levels (Mezquita *et al.,* 2000).

2.2
Regulators of Ang2 expression

Since it was discovered, investigators have found many factors and cytokines, which can modulate Ang2 mRNA and/or protein expression. One of the most important of these factors to be studied is hypoxia. In a 1998 study, Mandriota and Pepper found that hypoxia increased expression of Ang2 mRNA, by three-to fivefold (Mandriota and Pepper, 1998). These levels were also increased two-fold by vascular endothelial growth factor (VEGF). This finding was important because it showed that Ang2 is an important component of the angiogenic switch of a tumor from an avascular to a vascular phase. It also demonstrated that Ang2 and VEGF cooperate in the regulation of neovascularization of ischemic tissues. In another study concerning hypoxia, investigators found that it significantly increased Ang2 mRNA at 8 and 24 hours compared to normoxic controls. Inhibitors of tyrosine kinase and protein kinase C but not mitogen-associated-protein (MAP) kinase significantly reduced this stimulation (Yan *et al.,* 2000).

The induction of Ang2 by hypoxia is facilitated, in part, by hypoxia-inducingfactor-1 (HIF-1). Cyclohexidine, which inhibits HIF-1 activity, reduced basal Ang2 mRNA expression, but caused a significant induction of VEGF mRNA (Mandriota *et al.,* 2000). On the other hand, compounds that inhibit flavoprotein oxidoreductase mimicked the induction of Ang2 mRNA by hypoxia in cultured endothelial cells (EC), but had no effect on VEGF expression in normoxic cells (Mandriota *et al.,* 2000). Therefore, Ang2 expression is regulated in a different fashion than VEGF and involves oxidoreductase flavoprotein, as well as tyrosine kinase and protein kinase C second-messenger systems.

In another study demonstrating the importance of hypoxia in Ang2 expression, Beck *et al.,* (2000) studied ischemia-triggered angiogenesis using a model of middle cerebral artery occlusion (MCAO). Ang2 mRNA was upregulated 6 hours after MCAO and was mainly observed in endothelial cell cord tips in the peri-infarct area and infarct area. This upregulation reached a maximum 12 to 24 hours after MCAO and coincided with VEGF upregulation and endothelial cell proliferation. The endothelial cell proliferation was also preceded by a transient period of endothelial cell apoptosis, correlating with a change in VEGF/Ang2 balance. This finding of early Ang2 mRNA upregulation in the tips of endothelial cell cords is very similar to that seen at the invading front of the vascular sprouts in the developing corpus luteum.

Several cytokines have been implicated to play a role in Ang2 regulation. Two cytokines known to be important factors required for maturation and stabilization of blood vessels, TGF-β1 and Ang1, have been shown to downregulate Ang2 expression (Mandriota and Pepper, 1998). Kim *et al.,* (2000c) found that TNF-a upregulated Ang2 expression in a time-and dose-dependent fashion. Other cytokines that have been shown to upregulate Ang2 expression include VEGF, bFGF, angiotensin II, and thrombin. Mandriota and Pepper found that VEGF, bFGF, and hypoxia increase the expression of Ang2, suggesting that an increase in Ang2 expression is a common pathway by which different angiogenic inducers act (Mandriota and Pepper, 1998). Recently, investigators have also found that the expression of Ang2 mRNA was upregulated about 4-fold by thrombin in endothelial cells with a peak at 4 hours (Huang et al., 2002). This was inhibited by hirudin, a specific inhibitor of thrombin. Inhibitors of serine/threonine kinasess, reversed the upregulation, implicating these two systems in the thrombinmediated upregulation of Ang2.

Finally, Otani *et al.,* have found that angiotensin II stimulates Ang2 but not Ang1 mRNA expression in a dose-and time-dependent manner. This stimulation was completely inhibited by protein kinase C inhibitor and partially by MAPkinase inhibitor (Otani *et al.,* 2001). In another study, other investigators confirmed that angiotensin II significantly induced Ang2 mRNA accumulations without affecting Ang1 or Tie2 expression; inhibition of epidermal growth factor (EGF) receptor abolished the induction of Ang2 (Fujiyama *et al.,* 2001). They found that the angiotensin receptors 1 and 2 (ATl and AT2) differentially regulate Ang2 and VEGF expression. AT1 stimulates processing of heparin-binding EGF by metalloproteinases, which transactivates EGF-receptor to induce angiogenesis via the combined effects of Ang2 and VEGF On the other hand, AT2 attenuates these affects by blocking EGF-receptor phosphorylation (Fujiyama *et al.,* 2001).

2.3
Proposed biologic roles of Ang2

The original investigations of Ang2 revealed that it binds to the Tie2 receptor with similar affinity as Ang1 (Maisonpierre *et al.,* 1997). Unlike Ang1, it does not cause phosphorylation of Tie2, but does block Ang1 activation of Tie2. However, more recent studies seem to contradict this observation about Tie2 phosphorylation. Using a 3-D fibrin matrix, investigators studied Tie2 phosphorylation using recombinant Ang2. A brief exposure of endothelial cells to Ang2 did not induce significant Tie2 receptor phosphorylation. However, after a 24-hour pretreatment, followed by a brief re-exposure, there was a significant increase in Tie2 phosphorylation comparable to that seen with Ang1 (Teichert-Kuliszewska *et al.,* 2001). These authors also found that Ang2 produced a significant increase in endothelial cell proliferation and endothelial cell differentiation on 3-D matrices. One explanation for these findings is that brief increases in Ang2 expression may result in inhibition of Tie2, thus releasing the vascular endothelium from the constitutive homeostatic influence of Ang1 and clearing the way for endothelial cell activation. However, prolonged exposures may shift the effects of Ang2 more towards agonism and therefore contribute to tube formation and neovessel stabilization (Teichert-Kuliszewska *et al.,* 2001).

In tissue localization studies, Ang2 expression is abundant in areas of vascular remodeling. In normal adult tissue, Ang2 expression is readily detectable only in ovary, placenta and uterus. Also, in the rat ovary, Ang2 transcripts are abundant at the leading front of vessels invading the corpus luteum (Maisonpierre *et al.,* 1997). Other authors have found that Ang2 mRNA is more abundant in the lung compared to other tissues in normoxic rat tissues (Abdulmalek *et al.,* 2001). However, after 48 hours of hypoxia, these levels increased sevenfold in the cerebellum and decreased in the heart, lung, and diaphragm (Abdulmalek *et al.,* 2001). Thus, it appears that Ang2 expression in quiescent tissues is usually minimal but changes dramatically in pathologic conditions such as hypoxia that lead to vascular remodeling.

Most investigators believe that Ang2 plays a dynamic role in vascular remodeling and angiogenesis in conjunction with VEGF and Ang1. One interpretation is that in the presence of abundant VEGF, Ang2 can promote vessel sprouting by blocking a constitutive stabilizing Ang1 signal, whereas in the absence of VEGF, Ang2 inhibition of Ang1 leads to vessel regression (Maisonpierre *et al.,* 1997). There is some controversy as to whether tumors get their new blood supply by coopting existing blood vessels or by angiogenesis. In a study using a rat glioma model, Holash *et al.* (1999) found that even the smallest tumors at just 1 week after implantation (<1 mm) were well vascularized; this was attributed to the co-opting of existing brain blood vessels without evidence of angiogenesis. These vessels later regressed (marked by Ang2 in the absence of VEGF) by an apoptotic mechanism that may involve disrupted interactions between EC, the surrounding extracellular matrix, and supporting cells. By 4 weeks, the tumors were much larger with necrotic centers; however, there was robust angiogenesis at the tumor periphery. These angiogenic vessels continued to express Ang2, which suggests that the destabilizing action of Ang2 facilitates the angiogenic action of VEGF at the tumor rim.

Therefore, the angiogenic properties of tumor-derived VEGF may be facilitated by vessel destabilization due to Ang2.

However, in another more recent study, investigators used epifluorescence microscopy and multi-photon laser scanning confocal microscopy to visualize C6 microglioma vascularization. They show that multicellular aggregates (<1 mm^3) initiate vascular growth by angiogenic sprouting via the simultaneous expression of VEGF receptor-2 (VEGFR-2) and Ang2 by host and tumor endothelium. Host blood vessels were not co-opted by tumor cells but rather are used as trails for tumor cell invasion of host tissue (Vajkoczy *et al.,* 2002). They also used *in situ* hybridization to investigate the temporal expression patterns of Ang2, VEGFR, and VEGF. In day-3 tumors, VEGF mRNA levels were low, and there was no expression of VEGFR and Ang2. At 6 days, there was a strong simultaneous upregulation of Ang2 and VEGFR-2 mRNA in endothelial cells of tumor vessels and adjacent host vessels. The expression of Ang2 appeared to be restricted to a subset of VEGFR-2-positive EC. This is consistent with the hypothesis that Ang2 induction occurs only transiently in intact vessels and triggers the initial destabilization of blood vessels, whereas VEGFR-2 expression is characteristic of activated endothelium (Vajkoczy *et al.,* 2002).

By 2 weeks, tumors were fully vascularized and their microvasculature was undergoing continuous remodeling. At this stage, there was strong expression of Ang2 and VEGFR-2, and VEGF expression had increased significantly (Vajkoczy *et al.,* 2002). These results show a coordinated activity of Ang2 and VEGF that leads to an increase in host vessel permeability, loss of blood-brain-barrier function in cerebral vessels, microvascular dilation, and sprout formation. The VEGF/Ang2 balance determines whether the new tumor vessels will continue to expand (when the ratio of VEGF to Ang2 is high) or regress (when the ratio is low) during the remodeling of tumor microvasculature.

Interestingly, Ang2 overexpression can lead to failure of tumor angiogenesis because of the imbalance between VEGF, Ang1, and Ang2. Yu and Stamenkovic found that overexpression of Ang2 in Lewis lung carcinoma (LLC) and TA3 mammary carcinoma cells inhibited their ability to form metastatic tumors and prolonged the survival of these mice in a tail vein injection model (Yu and Stamenkovic, 2001). Tumors derived from Ang2-overexpressing cells displayed aberrant angiogenic vessels—vascular cords or aggregated vascular endothelial cells with few associated smooth muscle cells; these were accompanied by endothelial and tumor cell apoptosis (Yu and Stamenkovic, 2001).

In a subcutaneous tumor model, expression of Ang2 in LLC or TA3 resulted in marked reduction of tumor growth with tumors failing to grow larger than 1 to 3 mm after several weeks compared to control tumors which rapidly grew. Massive apoptosis, as indicated by TUNEL assay, was seen in both endothelial cells and tumor cells within the tumor centers (Yu and Stamenkovic, 2001). They concluded that the imbalance between VEGF, Ang1, and Ang2 created by tumor cells overexpression of Ang2 leads to disruption of tumor angiogenesis, which results in decreased local tumor growth and inhibition of tumor formation in metastasis assays. The mechanism may be secondary to the inability to recruit smooth muscle cells leading to the absence of fully formed blood vessels and lack of signals generated by physical interaction between smooth muscle cells and endothelial cells that ensure endothelial cell survival.

In conclusion, angiopoietin-2 is a novel cytokine that plays an important role in vascular remodeling. It binds to the tyrosine-kinase-specific receptor Tie2 and may induce phosphorylation of this receptor under certain conditions. It also has at least three different splice variants with different functions. It occupies an integral step in the interplay between several factors implicated in tumor angiogenesis, including hypoxia, basic fibroblast growth factor, vascular endothelial growth factor, tumor necrosis factor-alpha, thrombin, angiotensin II, tumor growth factor-beta1, and angiopoietin-1. There are several second-messenger systems that may play a role in its action on endothelial cells, including protein kinase C, mitogen-associated protein kinase, and serine/threonine kinases. Finally, the interaction between VEGF and angiopoietins-1 and-2 is essential for whether vasculature undergoes regression or sprouting.

3.
Angiopoietin 3 and 4

Using homology-based cloning, two additional angiopoietins have been isolated. Angiopoietin-3 and Angiopoietin-4 likely represent mouse and human versions of the same gene locus. They are more structurally divergent than the mouse and human counterparts of Ang-1 or Ang-2, sharing only 54% homology, while Ang-1 and Ang-2 share 99% and 87% respectively (Valenzuela *et al.,* 1999). These angiopoietins share the main structural characteristics of Ang-1 and Ang-2, including homology throughout the signaling peptide, N-terminal region, the coiled-coil segment, and the fibrinogen-like domain containing a pattern of three closely spaced cysteine residues. They also bind Tie-2.

The tissue distributions of Ang-3 and Ang-4 are also quite divergent. Ang-4 was prominently expressed only in human lung, with much lower levels in other tissues. Ang-3 was much more widely distributed. This difference in expression suggests different roles. Indeed, Ang-4 was able to activate Tie-2, while Ang-3 could not. Further, Ang-3 was able to limit the ability of Ang-1 to activate Tie-2. The discovery of additional angiopoietins suggests increased complexity in angiogenic regulation.

4.
Summary

Angiogenesis consists of a variety of cellular processes including matrix dissolution/remodeling, migration, branching, cell recruitment, and cell stability. The angiopoietins appear to be significantly involved in all of these listed activities. The mechanisms by which the angiopoietins regulate these activities and are themselves regulated are still not clear. However, growing evidence indicates that interactions with other angiogenesis factors, in addition to each other, are important mechanistic features. Furthermore, the identification of splice variants for angiopoietin 1 suggests a more complicated regulation of the angiopoietins. Continued research of this interesting gene family promises to provide novel insights into the angiogenesis process as well as reveal unique molecular paradigms in the regulation of cell behavior.

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4
The ETS family of transcription factors

Yasufumi Sato

1.
Introduction

Transcription factors determine gene expression by binding to the specific DNA sequences within the promoter regions, by forming complexes with co-regulatory proteins, and by allowing transcriptional activation or repression. The ETS (E twenty-six) family of transcription factors is defined by a conserved DNA-binding ETS "domain" that binds to a purine-rich consensus core sequence GGA(A/T) of ETS binding motif in the promoter or enhancer region of target genes (for a review, see Wasylyk *et al.,* 1993). ETS domain is composed of 85 amino acids and forms a winged helix-turn-helix structure with three α-helices and four fJ-sheets. At present, more than 50 proteins containing this ETS domain are identified in species ranging from drosophila to human, and they are divided into several subclasses according to the position of the ETS domain and the presence of specific subdomains. Most ETS family members activate gene transcription, while some such as ERF, NET, TEL and YAN act as transcriptional repressors (for a review, see Mavrothalassitis and Ghysdael, 2000). Regions beside the ETS domain also influence the function of the ETS family members. In at least seven ETS family members, two inhibitory regions flanking the ETS domain negatively regulate the binding to DNA. Especially, the N-terminal flanking region is responsible for inhibition of DNA binding by interacting with ETS domain and with the C-terminal flanking region. This inhibition is disrupted by the unfolding of an α-helix in ETS domain, which renders the binding of ETS domain to DNA. Pointed domain, which is homologous to a drosophila ETS family member Pointed, is also conserved in a number of ETS family members. Pointed domain contains target amino acid sequences for MAP kinase, and once these sequences are phosphorylated by MAP kinase, transactivation activity of ETS protein is markedly enhanced (for reviews, see Papas *et al.,* 1997; Sharrocks, 2001).

Individual ETS family members are expected to regulate the expression of distinct target genes thereby generating functional specificity. This specificity is derived from the regulation of the ETS family members at several different points. Individual ETS family members display specific recognition of DNA binding sites. DNA sequence of the flanking region of ETS binding motif is variable, which determines the specific DNA recognition of individual ETS family members. The ETS family members are known to bind to various

partner proteins, and this protein-protein interaction with partner proteins also plays a significant role in the targeting of the ETS family member to specific DNA binding sites. The expression pattern of individual ETS family members is different depending on the various cell types, which determines the cell-type specific function. Different ETS family members have distinctive responses to intracellular signal transduction pathways. Accordingly, the function of each ETS family member is regulated by a combination of various mechanisms that would allow the control of specific gene regulation (for reviews, see Graves and Petersen, 1998; Li *et al.,* 2000; Wasylyk *et al.,* 1998). Indeed, targeted disruption of the ETS family member genes confirms that individual ETS family members have unique biological properties (for a review, see Bartel *et al.,* 2000). However, the phenotypes observed on gene disruption do not always correlate with exact expression pattern of the ETS family members. These findings may suggest some functional redundancy within members of this family.

2.
Expression and possible involvement of the ETS family of transcription factors in embryonic vascular development

The vascular system is the first functional organ that develops in the embryo. The mesodermal stem cells, so-called hemangioblasts, aggregate and form blood islands in the extraembryonic yolk sac, where they differentiate into an external layer of endothelial cells (ECs) and an inner core of blood cells. These outer ECs constitute the primary vascular plexus. Similarly, hemangioblasts and/or angioblasts located in the intraembryonic proximal lateral mesoderm differentiate into ECs and organize the dorsal aorta. These processes of *in situ* vascular formation are called vasculogenesis. Subsequently, neo-vessels are generated from the primary vascular plexus by sprouting and intussusception and become distributed throughout the entire body. This process is called angiogenesis. In the final process of vascular development, interstitial mesenchymal cells differentiate into mural cells (smooth muscle cells and pericytes), surround blood vessels, and make the vessels mature and stable (for review, see Risau, 1997).

Spatial and temporal coordination in the expression of various genes is required for the development of various organs (differentiation and morphogenesis), and transcription factors play fundamental roles in these processes. A variety of transcription factors are expressed in endothelial cells (ECs), mural cells and their progenitors during the period of vascular development in embryo. It is evident that the ETS family of transcription factors is involved in a diverse array of biological functions including cellular differentiation as well as organ morphogenesis (for a review, see Maroulakou and Bowe, 2000). As will be described in the following, ETS-1, ERG, FLI-1 and TEL in the ETS family members are expressed in endothelial lineage during vascular development. Complexity lies in the fact that plural ETS family members are simultaneously expressed

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in ECs or its progenitors during vascular development, and thus the functions of the ETS family of transcription factors may be sometimes overlapped.

2.1
ETS-1

ETS-1 was first identified as the cellular progenitor of the viral oncogene *v-ets* in the genome of the avian leukemia retrovirus E26, and is the prototype of the ETS family of transcription factors (Leprince *et al.,* 1983; Nunn *et al.,* 1983). ETS-1 is the first member of the ETS family members, which is expressed in mesoderm lineage cells including ECs during embryogenesis (Vandenbunder *et al.,* 1989). Maroulakou *et al.* (1994) compared the patterns of expression of ETS-1 and its closely related ETS-2 during murine embryogenesis, and found that ETS-1 was preferentially expressed in developing vascular structures, including the heart, arteries, capillaries, and meninges. In relation to the relatively specific expression pattern of ETS-1 in ECs in embryo, an EC-specific element for expression of ETS-1 has been identified in the first intron of the *ets-1* gene (Jorcyk *et al.,* 1997). ETS-1 (-/-) mice were viable and fertile, but displayed about 50% perinatal mortality (Barton *et al.,* 1998). However, the cause of this premature mortality is not known.

2.2
FLI-1 (Friend leukemia int integration-site 1)

Shortly after the evaluation of ETS-1 in the embryonic vascular system, other members of the ETS family members were found to be expressed in ECs and their progenitors in embryo. FLI-1 is closely related to the occurrence of erythroleukemia, whose locus is disrupted in Friend murine leukemia virus (BenDavid *et al.,* 1990). The expression of FLI-1 was shown in hemangioblasts, angioblasts, and ECs in embryo (Melet *et al.,* 1996; Meyer *et al.,* 1995). More recently, Brown *et al.,* (2000) examined the expression pattern of Zebrafish FLI-1. Its expression was initially observed in the posterior lateral mesoderm, overlapping with that of GATA-2 in a potential hemangioblast population. GATA-2 belongs to the GATA family of transcription factors, and is a marker of hematopoiesis. Subsequently, FLI-1 and GATA-2 expression patterns diverged, with separate FLI-1 and GATA-2 expression domains arising in the developing vasculature and sites of blood formation, respectively. Therefore, FLI-1 is one of the earliest indicators of hemangioblast formation.

The function of FLI-1 was further examined by gene targeting in mice. FLI-1 (-/-) murine embryos were able to form a functional network of blood vessels, indicating that vasculogenesis and angiogenesis could proceed without this transcription factor. However, FLI-1 (-/-) murine embryos died at embryonic day 11.5 to 12.5, with a loss of vascular integrity leading to cerebral hemorrhage (Hart *et al.,* 2000; Spyropoulos *et al.,* 2000).

2.3
ERG (ETS-related gene)

ERG is closely related to FLI-1, and is encoded on chromosome 21 (Rao *et al.,* 1987). Vlaeminck-Guillem *et al.* (2000) recently reported that the *erg* gene was expressed predominantly in mesodermal tissues, including ECs, precartilaginous and urogenital areas, as well as in migrating neural crest cells in mouse embryo. Thus, the expressions of ERG in ECs are overlapped with its closely related FLI1. ERG knockout mice have not been reported to date. However, ERG and FLI-1 may play distinctive roles, since ERG was not able to compensate the function of FLI-1 in FLI-1 (-/-) murine at least in the development of vascular system. On the other hand, Baltzinger *et al.* (1999) reported that the ectopic expression of Xenopus ERG in the ventral region of Xenopus embryos induced the ectopic endothelial differentiation at the site of injection. Thus, this gain-of-function experiment suggested the possible involvement of ERG in vascular development.

2.4
TEL (translocated ETS leukemia)

TEL is a sequence-specific transcriptional repressor of ETS-driven transcription (Golub *et al.,* 1994; Kwiatkowski *et al.,* 1998; *Lopez et al.,* 1999; Poirel *et al.,* 1997). TEL binds several ETS family members including FLI-1 through its Pointed domain and recruits co-repressors such as mSlNBA, N-COR and SMRT (Chakrabarti *et al.,* 1999; Fenrick *et al.,* 1999; *Lopez et al,* 1999).

TEL is widely expressed in various cells including endothelial lineage throughout embryonic development (Lopez *et al.,* 1999; Wang *et al.,* 1997). TEL (-/-) murine embryos exhibited normal vasculogenesis but defective angiogenesis in yolk sac, and intraembryonic apoptosis of mesenchymal and neural cells (Wang *et al.,* 1997). Thus, TEL is thought to be required for angiogenesis in the yolk sac and for survival of selected cell types within the embryo proper. However, the exact targets of TEL for transcriptional repression in those processes remain to be elucidated.

3.
Role of ETS-1 in postnatal angiogenesis

In the postnatal state, blood vessels, which are composed of ECs and mural cells, are normally stabilized. Thus, postnatal angiogenesis includes at least six sequential steps: (i) detachment of pre-existing pericytes for vascular destabilization (pericyte drop-off); (ii) extracellular matrix (ECM) degradation by endothelial proteases; (iii) migration of Ecs; (iv) proliferation of Ecs; (v) tube formation by Ecs; and (vi) reattachment of pericytes for vascular restabilization/maturation.

Hypoxia is known to be one of the most important triggers of angiogenesis. Hypoxia induces the expression of vascular endothelial growth factor (VEGF), one of the most important angiogenic factors, which acts on ECs mainly in a paracrine manner. Moreover, hypoxia induces angipoietin-2 (Ang-2), an antagonistic ligand of the endothelial TIE-2

receptor, in various cell types including ECs. Ang-2 antagonizes the agonistic ligand angiopoietin-1 (Ang-1) of TIE-2, resulting in pericyte drop-off and vascular destabilization. Consecutively, VEGF stimulates ECs of destabilized vessels and forms neo-vessels by augmenting protease synthesis for ECM degradation, migration, and proliferation. Finally, immature neo-vessels composed of ECs become surrounded by pericytes, a process determined by the balance of Ang-1 and Ang-2. Eventually, Ang-1 dominates over Ang-2 and stimulates the reattachment of pericytes for vascular restabilization. In addition, Ang-1 can directly stimulate the migration of ECs. When pericytes reattach to ECs of neo-vessels in this manner, latent transforming growth factor-β (TGF-β) is efficiently activated, causing maturation of the neovessels *(Figure 1)* (for a review, see Hanahan, 1997).

A variety of transcription factors are expressed in ECs during angiogenesis, and they regulate the properties of ECs. Among the ETS family of transcription factors, ETS-1 is the best-characterized factor involved in angiogenesis.

3.1
Expression of ETS-1 in ECs during angiogenesis

Increased expression of ETS-1 was observed in ECs of neo-vessels during tumor angiogenesis (Wernert *et al.,* 1992, 1994) as well as in those cells participating in angiogenesis during the healing of gastric ulcers (Ito *et al.,* 1999). When large vessels are denuded, the remaining ECs at the wound edge migrate, proliferate, and repair the denuded area. This process, termed "re-endothelialization", resembles angiogenesis and provides an important mechanism for maintaining the normal vascular wall. Recently we observed that ETS-1 was inducibly expressed in ECs at the edge of the rat aorta after denuding injury (Tanaka *et al.,* 1998).

The expression of ETS-1 in ECs was shown to be induced by representative angiogenic growth factors such as VEGF, acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF), and epidermal growth factor (EGF) (Iwasaka *et al.,* 1996). The induction of ETS-1 in ECs by either VEGF or bFGF was inhibited by a specific MEK1 inhibitor, indicating that the MEK1-ERK1/2 pathway was required for this induction (Kanno *et al.,* 2000; Sato *et al.,* 2000; Tanaka *et al.,* 1999). Especially, in the case of VEGF-stimulated induction, VEGF receptor 2 (VEGFR-2/KDR)-mediated activation of ERK1/2 was responsible for this induction (Kanno *et al.,* 2000; Sato *et al.,* 2000).

3.2
Effect of hypoxia on the expression of ETS-1

Hypoxia is closely related to angiogenesis, and it induces angiogenesis-related molecules such as VEGF. Hypoxia activates hypoxia inducible factor 1 (HIF-1), a basic helix-loop-helix (bHLH)/Per-AHR-ARNT-Sim (PAS) transcription factor. HIF-1 is a heterodimer transcription factor consisting of a constitutively expressed HIF-1β/ARNT (aryl hydrocarbon receptor nuclear translocator) subunit and an O_2-regulated HIF-lα subunit,

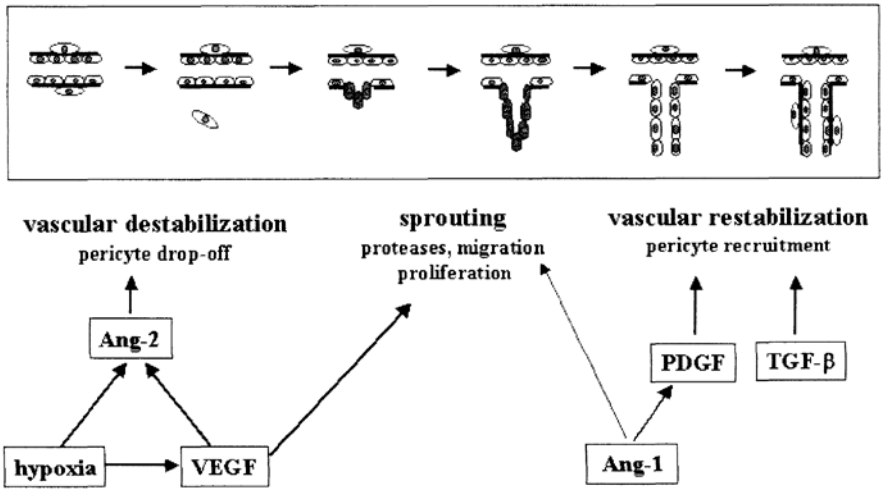

Figure 1. Angiogenesis in adult. Angiogenesis includes following sequential steps: (i) detachment of pre-existing pericytes for vascular destabilization (pericyte drop-off); (ii) extracellular matrix (ECM) degradation by endothelial proteases; (iii) migration of Ecs; (iv) proliferation of Ecs; (v) tube formation by Ecs; and (vi) reattachment of pericytes for vascular restabilization/maturation. The balance of various factors including VEGF, Ang1, Ang-2, PDGF and TGF-β regulates these processes.

and the activation of HIF-1 depends primarily on redox-sensitive stabilization of HIF-1α (Huang *et al.,* 1996; Wang and Semenza, 1995).

We showed that hypoxia strongly induced the expression of ETS-1 (Oikawa *et al.,* 2001). We further disclosed that the consensus core sequence of the hypoxia responsive element, ACGT, was located between –424 and –279 of the human ETS-1 promoter, and that this element was responsible for the hypoxia-mediated induction of ETS-1. Thus, this interaction betwenn ETS-1 and HIF-1 is expected to play a role in angiogenesis.

3.3
Role of ETS-1 in angiogenesis

As described above, ETS-1 is expressed in ECs during angiogenesis. Moreover, the specific elimination of ETS-1 expression in ECs by *ets-1* antisense oligodeoxynucleotides (ODNs) inhibited expression of uPA and MMP-1, migration and tube formation *in vitro* (Iwasaka *et al.,* 1996), and a non-proliferative adenovirus encoding dominant negative ETS-1 inhibited *in vivo* angiogenesis (Nakano *et al.,* 2000). These results indicate that ETS-1 in ECs is indeed required for angiogenesis.

In order to understand the role of ETS-1 in angiogenesis, we transfected murine ECs with *ets-1* sense cDNA or antisense cDNA and established high or low ETS-1 expression EC lines, and compared the angiogenic properties of these cell lines with those of the parental murine EC line. The growth rate was almost identical for each cell line, indicating that

ETS-1 did not play a major role in proliferation. In contrast, the invasiveness was markedly enhanced in high ETS-1 expression cells and reduced in low ETS-1 expression ones compared with that of the parental cells. During sprouting angiogenesis, ECs inducibly express proteases for matrix degradation and integrin αvβ3 for cell migration, and thereby invade into the interstitial space. The gene expressions of matrix metalloproteinases (MMP-1, MMP-3, and MMP-9) as well as the gelatinolytic activity of MMP-9 were significantly increased in high ETS-1 expression cells, whereas low ETS-1 expression cells exhibited reduced expression of the integrin β3 subunit and impaired adhesion to vitronectin (Oda *et al.,* 1999). These results indicate that ETS-1 is an important regulator, converting ECs to the invasive phenotype.

3.4
Target genes of ETS-1 in ECs

Endothelium-specific angiogenesis-related receptor-type tyrosine kinases such as VEGFR-1 (Flt-1), VEGFR-2 (FLK-1/KDR), TIE-1, and TIE-2 contain the ETS binding motif in their promoter/enhancer regions (Dube *et al.,* 1999; Iljin *et al.,* 1999; Kappel *et al.,* 2000; Wakiya *et al.,* 1996). Indeed, significant correlations were found between the expression of ETS-1 and that of VEGFR-1 or VEGFR-2 in ECs *in vivo* (Kappel *et al.,* 2000; Valter *et al.,* 1999). By using adenovirus-mediated gene transfer in human umbilical vein ECs (HUVECs), we observed that wild-type ETS-1 increased, and dominant negative ETS-1 decreased, the protein levels of VEGFR-2 and TIE-2 (Fig. 2). In the case of VEGFR-1, wild-type ETS-1 did not significantly affect the protein level of VEGFR-1, but dominant negative ETS-1 did decrease it (Fig. 2). Therefore, although ETS-1 does not exhibit a strong transactivation activity on VEGFR-1, it seems to be permissive for the expression of VEGFR-linHUVECs.

To further clarify the downstream targets of ETS-1, we transiently overexpressed ETS-1 in HUVECs and comprehensively searched for potential downstream targets of ETS-1 by use of cDNA microarray analysis. This analysis disclosed that the expression of seven angiogenesis-related genes, namely podocalyxin-like, ephrin-A1, MMP-1, Ang-2, calreticulin, neuropilin-1, and uPA receptor, was augmented more than 2.5 fold by ETS-1 in HUVECs. Of these, MMP-1 is the only molecule that has already been recognized as a downstream target of ETS-1 in ECs, whereas the other six genes have not been previously recognized as downstream targets of ETS-1 (Teruyama *et al.,* 200la).

Neuropilin-1 was initially identified as a membrane protein expressed in developing neurons, and was shown to function as a receptor for the class 3 semaphorins, which produces inhibitory axon guidance signal (for a review, see Fujisawa and Kitsukawa, 1998). However, it became evident that neuropilin-1 was also expressed in ECs, where it functioned as a co-receptor of VEGFR-2 for $VEGF_{165}$ and several other members of the VEGF family (for a review, see Miao and Klagsbrun, 2000). Neuropilin-1 mutant mouse embryos exhibited severe abnormalities in cardiovascular system (Kawasaki *et al.,* 1999), indicating that neuropilin-1 plays important roles in embryonic nervous tissue and vessel formation. Moreover, neuropilin-1 is thought to be involved in postnatal angiogenesis, since neuropilin-1 expression was augmented in the tissues of patients with rheumatoid

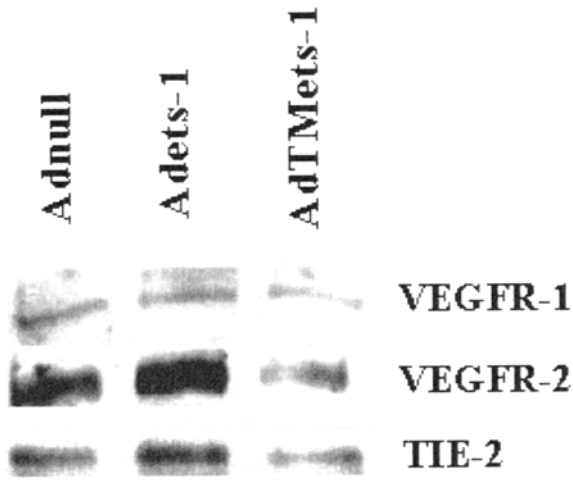

Figure 2. Effect of ETS-1 on the protein levels of VEGFR-1, VEGFR-2, and TIE-2 in HUVECs. HUVECs were infected with Adnull, Adets-1 or AdTMets-1 (200 m.o.i.). After a 48-hour incubation, protein was extracted, and Western blotting for VEGFR-1, VEGFR-2, and TIE-2 was performed.

arthritis or diabetic proliferative retinopathy, and this expression correlated with that of VEGFR-2 (Ikeda *et al.,* 2000; Ishida *et al.,* 2000). Further study is required to clarify the promoter region of the neuropilin-1 gene to determine whether or not an ETS binding site (s) exists and functions there. *Table 1* summarizes the possible target molecules of ETS family members in ECs for the regulation of angiogenesis.

3.5
Regulation of ETS-1 activity

As described above, ETS-1 is thought to be responsible for the initiation and progression of angiogenesis. However, at the time of vascular restabilization/maturation, the transactivation activity of ETS-1 in ECs needs to be terminated. Vascular restabilization/maturation is characterized by pericyte reattachment, and TGF-β is thought to be involved in this process (Folkman and D'Amore, 1996). TGF-βis a 25-kDa homodimeric polypeptides that is always secreted in an inactive form. Latent TGF-p (LTGF-β) is a complex of mature TGF-β, the TGF-β latency-associated peptide (LAP), and the latent TGF-β binding protein (LTBP) (Miyazono *et al.,* 1988). LTGF-β is efficiently activated when pericytes attach to ECs, and this activation of LTGF-β is dependent on the plasminogen activator-plasmin system, which is responsible for ECM degradation during angiogenesis (Sato and Rifkin, 1989; Sato *et al.,* 1990).

We examined the effect of TGF-β on the transactivation activity of ETS-1. TGF-β augmented mRNA expression and protein synthesis of ETS-β. However, when the DNA binding activity and the promoter activity were examined, TGF-β attenuated the DNA-ETS complex formation and the promoter activity driven by ETS-1 (Yagi-Iwasaka *et al.,*

Table 1. Possible targets of ETS family members in ECs for the regulation of angiogenesis

Target	Reference
VEGFR-1 (Flt-1)	Wakiya *et al.*, 1996
VEGFR-2 (KDR/Flk-1)	Iljin *et al.*, 1999
Neuropilin-1	Teruyama *et al.*, 2001a
TIE-1	Dube *et al.*, 1999
TIE-2	Kappel *et al.*, 2000
Ang-2	Teruyama *et al.*, 2001a
u-PA	Iwasaka *et al.*, 1996
MMP-1	Iwasaka *et al.*, 1996, Oda *et al.*, 1999
MMP-3	Oda *et al.*, 1999
MMP-9	Oda *et al.*, 1999
integrin β3 subunit	Oda *et al.*, 1999
VE-cadherin	Lelievre *et al.*, 2000
Heme oxygenase 1	Dermaudt *et al.*, 1999
Endothelin converting enzyme 1β	Orzechowski *et al.*, 1998

2001). This inhibitory effect of TGF-β required new protein synthesis, although the exact nature of the protein remains to be elucidated. Thus, when latent TGF-βis activated at the time of vascular restabilization/maturation, TGF-β is thought to attenuate the transactivation activity of ETS-1 of ECs by inducing a protein that interferes with the binding of ETS-1 to its DNA binding site.

4. ETS-1 and endothelial apoptosis

Apoptosis is a term used to describe the terminal morphological and biochemical events that occur in programmed cell death. Apoptosis of endothelial cells (ECs) was observed at the initiation of angiogenesis, at the stage of branching of or communication among newly formed vessels, and at the regression of neo-vessels (for reviews, see Nor and Polverini, 1999). The ETS family of transcription factors including ETS-1 are involved in apoptosis. Especially, targeted elimination of the *ets-1* gene caused apoptosis of T cells and natural killer cells (Barton *et al.,* 1998; Bories *et al.,* 1995; Muthusamy *et al.,* 1995). In contrast, p42-ETS-1, a splicing variant of ETS-1 lacking inhibitory domain for DNA binding and thus a stronger transcription factor than wild-type p51-ETS-1, induced apoptosis of human colon cancer cells (Huang *et al.,* 1997). Thus, ETS-1 can be either anti-apoptotic or pro-apoptotic.

We examined whether ETS-1 played any role in the apoptosis of ECs (Teruyama *et al.,* 2001b). HUVECs become apoptotic when they are cultivated under serum-free conditions. Transfection of HUVECs with the wild-type *ets-1* gene increased the number of apoptotic cells in a serum-free environment. VEGF exhibited an anti-apoptotic effect as well as induced ETS-1 in ECs (Iwasaka *et al.,* 1996; Teruyama *et al.,* 2001b), and transfection of HUVECs with a dominant negative *ets-1* gene transfection further augmented the anti-

apoptotic effect of VEGF (Teruyama *et al.,* 200Ib). Thus, endogenously expressed ETS-1 is pro-apoptotic, counteracting the anti-apoptotic effect of VEGF. To clarify the mechanism by which ETS-1 promoted the apoptosis of HUVECs, we examined the pattern of expression of various genes related to apoptosis. It turned out that, besides up-regulating ang-2, ETS-1 was responsible for upregulation of pro-apoptotic genes such as Bid, cytochrome p450, caspase-4, p27, and p21 more than 2 fold, and for down-regulation of anti-apoptotic genes such as DAD-1, AXL, Cox-2, IAP-2, and MDM-2 less than 0.5 fold in HUVECs (Teruyama *et al.,* 2001b). Thus, overexpression of ETS-1 on the initiation of angiogenesis may be responsible for the localized apoptosis of ECs at the initiation of angiogenesis.

5. Concluding remarks

Angiogenesis is a complex phenomenon, one that requires migration, proliferation, differentiation, and morphogenesis. A number of molecules are expressed in ECs during angiogenesis. Thus, transcriptional regulation of gene expression in ECs has become an important issue for understanding the molecular mechanisms of angiogenesis. Increasing evidence suggests that the ETS family of transcription factors plays an important role in angiogenesis. However, our understanding about them is still limited. Thus, further study is required to clarify fully the role of the ETS family of transcription factors in the biology of ECs.

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5
Post-transcriptional regulation of VEGF

Gregory J.Goodall, Leeanne S.Coles, M.Antonetta Bartley and Kenneth J.D.Lang

1. Introduction

The growth and function of all tissues requires the establishment of an adequate vascular structure. In its absence the tissue becomes deprived of oxygen and nutrients, which cells respond to by producing angiogenic factors that function to recruit new blood vessels into the under-vascularized tissue (Carmeliet 2000; Risau 1997; Saaristo *et al.,* 2000). Of the numerous growth factors that contribute to angiogenesis, vascular endothelial growth factor (VEGF) plays a particularly crucial role as a key regulator of angiogenesis (Kim *et al.,* 1993; Millauer *et al.,* 1994; Shweiki *et al.,* 1992). The importance of VEGF during development, and the requirement for appropriate levels of its expression is demonstrated by the fact that mice bearing a single normal allele for the VEGF gene (i.e. heterozygous for VEGF gene knockout) die *in utero* due to a failure in vascular development (Carmeliet *et al.,* 1996; Ferrara et al., 1996).

VEGF is produced by most cell types, including fibroblasts, smooth muscle cells (Tischer *et al.,* 1991), epithelial cells (Breier *et al.,* 1992), peripheral blood mononuclear cells (Renner and Pilger 1999) and endothelial cells (Bocci *et al.,* 2001). It is highly expressed in solid tumors and in areas of active vascularization (Leung *et al.,* 1989; Plate *et al.,* 1992; Shweiki *et al.,* 1992). VEGF is a secreted, endothelial cell-specific mitogen that acts through two receptor tyrosine kinases, VEGFR-1 (previously termed Flt-1) and VEGFR-2 (previously Flk-1/KDR), which are expressed primarily on vascular endothelial cells (reviewed in Neufeld *et al.,* 1999). In addition to its mitogenic effect on endothelial cells, VEGF induces vascular permeability and has alternatively been called vascular permeability factor (VPF).

Increased expression of VEGF appears to be a critical step in the process of neovascularization of solid cancers (Kim *et al.,* 1993; Shweiki *et al.,* 1992, 1995). Inhibition of VEGF activity *in vivo* can block both tumor establishment and progression, by inhibiting vascularization (Kim *et al.,* 1993; Millauer *et al.,* 1994). VEGF also appears to be required for the maintenance of tumor blood vessels, as withdrawal of VEGF leads to breakdown of the vascular structure and consequently tumor regression (Benjamin and Keshet, 1997). VEGF has been intensely studied not only because of its role in tumor growth, but also as a potential mediator of "therapeutic angiogenesis", to promote the

growth of collateral blood vessels to supplement the blood supply to tissues affected by vascular disease (Freedman and Isner, 2002; Schratzberger *et al.,* 2001).

The expression of VEGF is subject to regulation at many levels (summarized in *Table 1*). The multitudinous levels of regulation are perhaps testament to the crucial role VEGF plays in tissue homeostasis, but it is interesting that despite the many potential ways that VEGF expression can be influenced, their combined influences cannot compensate for the halving of gene copy number in the heterozygous VEGF knockout mouse (Carmeliet *et al.,* 1996; Ferrara *et al.,* 1996). Perhaps not all the regulatory mechanisms are operational at certain crucial stages of development. Alternatively, the failure to compensate may be due to a requirement at times during embryogenesis for maximal levels of VEGF synthesis, when the inducing systems are fully activated. Under these circumstances the level of synthesis in the heterozygote will be half that in the normal embryo.

Table 1. Modes of regulation of VEGF expression

Mode of regulation	References
Alternative sites of transcription initiation	Akiri *et al.*, 1998
Alternative splicing	Bacie *et al.*, 1995; Ng *et al.*, 2001; Watkins *et al.*, 1999
mRNA stability	Stein *et al.*, 1995; White *et al.*, 1995; Ikeda *et al.*, 1995; Levy *et al.*, 1996b
Translation	Stein *et al.*, 1998
Proteolytic cleavage	Houek *et al.*, 1992; Plouet *et al.*, 1997

A number of cytokines have been found to elicit VEGF production, but the major inducer in growing tumors is thought to be oxygen and glucose deprivation (Shweiki *et al.,* 1995). Studies investigating regulatory mechanisms have focused mainly on hypoxia as the inducing stimulus. The up-regulation of VEGF mRNA level by hypoxia is due to both transcriptional activation and a marked stabilization of the normally labile VEGF mRNA (Damert *et al.,* 1997; Ikeda *et al.,* 1995; Levy *et al.,* 1995,1996b; Shima *et al.,* 1995;Stein *et al.,* 1995). In addition, the presence of an internal ribosome entry site (IRES) in the VEGF transcript is believed to contribute to its expression during hypoxia (Stein *et al.,* 1998). This chapter focuses on post-transcriptional aspects that affect VEGF expression. Transcriptional activation of VEGF in response to hypoxia is addressed in Chapter 6.

2. VEGF isoforms resulting from alternative splicing

Three major and several minor isoforms of the VEGF protein are generated as a result of alternative splicing of the pre-mRNA (*Figure 1*). The ratios of the isoforms change during

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development, and can vary between tissues or cell types, suggesting that regulation of the pattern of alternative splicing may occur to alter or fine tune the functional consequences of VEGF gene expression.

2.1
Major VEGF isoforms

The three major VEGF isoforms in humans have 121, 165 and 189 amino acids respectively, after removal of the 26 amino acid signal peptide (Leung *et al.,* 1989; Tischer *et al.,* 1991). Each isoform is expressed as a disulfide-linked homodimer. Human VEGF has an additional amino acid (Gly6) compared to most other mammals, so the major isoforms in the mouse and other animals are $VEGF_{120}$, $VEGF_{164}$ and $VEGF_{188}$. The isoforms all contain exons 1 to 5 and the terminal exon (exon 8). Thus all isoforms that result from alternative splicing have the same 5' and 3' untranslated regions, and consequently would be equally subject to post-transcriptional regulation mediated by these untranslated regions. (The potential for alternative lengths of the untranslated regions as a result of alternative polyadenylation sites is discussed later in this chapter.)

The regions encoded by exon 7 (which is present in $VEGF_{165}$ and $VEGF_{189}$) and exon 6 (which is present in $VEGF_{189}$) are rich in basic residues, and confer an affinity for heparan sulfate, a polyanionic constituent of the extracellular matrix. Consequently, whereas $VEGF_{121}$ freely diffuses when secreted from cells, $VEGF_{165}$ and especially $VEGF_{189}$ tend to remain bound near the surface of the secreting cell (Houck *et al.,* 1992; Park *et al.,* 1993). Proteolytic cleavage of $VEGF_{189}$ appears to be required both for its release from the extracellular matrix and for conversion to an active form that can bind both VEGF receptors (Houck *et al.,* 1992; Plouet *et al.,* 1997). In addition to having heparin-binding activity, $VEGF_{165}$ differs biologically from $VEGF_{121}$ due to differences in the types of receptor complex that it binds (Oh *et al.,* 2002; Whitaker *et al.,* 2001).

2.2
Minor isoforms

A minor form of VEGF that is 145 amino acids in length is found in placental and uterine tissues (Charnock-Jones *et al.,* 1993) and in cell lines derived from carcinomas of the female reproductive system (Poltorak *et al.,* 1997), while an isoform of 206 amino acids has been found in human cells (Houck *et al.,* 1991), but may not be present in the mouse (Shima *et al.,* 1996). A cDNA clone encoding an isoform of 115 amino acids with an alternative C-terminus has been isolated from a murine cell line (Sugihara *et al.,* 1998). In this isoform exon 4 is extended and exons 5, 6 and 7 are skipped (unpublished observation).

2.3
Differential expression and functions of the isoforms

Several studies indicate that the ratios of the different VEGF isoforms vary, depending on the tissue type (Bacic *et al.,* 1995; Ng *et al.,* 2001) or developmental stage (Ng *et al.,* 2001;

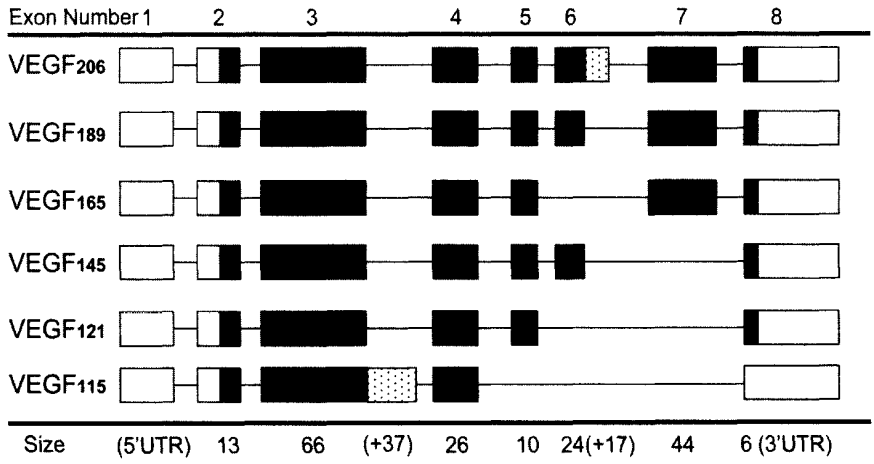

Figure 1. VEGF transcripts generated by alternative splicing. Alternatively spliced mRNA transcripts generating VEGF isoforms of 121,145,165,189 and 206 amino acids (after removal of the 26 amino acid signal peptide) have been detected in human cells or tissues (Tischer *et al.,* 1991), while an additional isoform of 115 amino acids has been detected in mouse cells and tissues (Sugihara *et al.,* 1998; Leung *et al.,* 1989; Houck *et al.,* 1991). The number of amino acids encoded by each exon is shown.

Watkins *et al.,* 1999), suggesting that the pattern of alternative splicing may be regulated. $VEGF_{164}$ is the predominant form in many tissues and cell lines, but in the spleen is equaled in abundance by $VEGF_{120}$, while in the heart and lung $VEGF_{188}$ is the most abundant form (Bacic *et al.,* 1995). A number of reports indicate distinct ratios of isotype expression are associated with particular diseases, including osteoarthritis (Pufe *et al.,* 2001b), rheumatoid arthritis (Ikeda *et al.,* 2000; Pufe *et al.,* 200la), diabetic nephropathy (Bortoloso *et al.,* 2001) and hyperoxic lung injury (Watkins *et al.,* 1999), or correlate with prognosis for various types of tumor (Chen *et al.,* 2000; Lee *et al., 1999*; Tomisawa *et al.,* 1999; Yuan *et al.,* 2001).

The functional implications of the regulation of isotype expression are not completely elucidated but some information on the consequences of severe changes in isoform ratios is available from gene replacement studies. Mice that express only the $VEGF_{120}$ isoform, due to specific removal of exons 6 and 7, suffer internal bleeding and impaired myocardial angiogenesis, resulting in death within a few hours or days after birth (Carmeliet *et al.,* 1999). $VEGF_{120}$ in these mice was expressed at a level equivalent to the sum of $VEGF_{120}$, $VEGF_{164}$ and $VEGF_{188}$ in normal mice, indicating that $VEGF_{164}$ and/or $VEGF_{188}$ have functions that cannot be replaced by $VEGF_{120}$. Further evidence for distinct functions of the major isoforms comes from a study of the growth and vascularization of tumors engineered to express each of the major isoforms (Grunstein *et al.,* 2000). Tumors expressing only $VEGF_{120}$ recruited vessels from the surrounding tissue to the surface of the tumor but were poorly vascularized internally. Tumors expressing $VEGF_{188}$ alone had a large number of small vessels within the tumor but failed to recruit vessels from

neighboring tissues. The vascularization of tumors expressing $VEGF_{164}$ alone resembled that of tumors from cells containing the wild-type VEGF gene. The different effects of the isoforms are consistent with the diffusibility of the isoform having a major effect on function. Interestingly, larger tumors were produced when cells expressing all three isoforms were mixed, than the tumors produced from mixtures of any two isoforms, suggesting a functional cooperation of the isoforms. There have been no reports to date on the molecular mechanisms involved in regulating the splicing pattern of VEGF transcripts.

3.
Regulation of VEGF mRNA stability

3.1
Rapid degradation of the VEGF mRNA

In unstressed cells the VEGF mRNA is labile, with a half-life of less than one hour (Ikeda et al., 1995; Levy *et al.,* 1996b; Stein *et al.,* 1995; White et al., 1995). Rapid turnover of the mRNA allows for rapid changes in expression of the gene product in response to changes in the cellular environment. Rapid mRNA turnover is a common feature of cytokines and growth factors that are secreted from cells, as is the case with VEGF. This is no doubt because other modes of post-transcriptional regulation that intracellular proteins may be subject to, such as phosphorylation or ubiquitination of the protein, are not available to the secreted proteins.

The instability of the VEGF mRNA is due in part to the presence of AU-rich elements (AREs) in the 3'UTR. AREs are present in the 3'UTRs of numerous cytokine, growth factor and immediate-early response genes, and target these mRNAs to be rapidly degraded by a mechanism that involves an accelerated removal of the poly(A) tail (Lagnado *et al.,* 1994; Shyu *et al.,* 1991; Wilson and Treisman 1988). The AREs in cytokine mRNAs typically contain multiple copies of a nonameric consensus sequence UUAUUUA(U/A)(U/A) (Chen and Shyu 1995; Lagnado *et al.,* 1994; Zubiaga *et al.,* 1995). The VEGF 3'UTR contains two perfect copies of this motif as well as four copies with a mismatch at a nonessential position. The nonameric elements in the VEGF 3'UTR are rather atypical in that they are neither closely clustered nor embedded in more extensive U-rich regions. Nevertheless, studies using a reporter gene containing insertions of various regions from the VEGF 3'UTR indicate that the VEGF AREs contribute to mRNA instability (Dibbens and Goodall, unpublished). In addition to the AREs that are present at several locations in the VEGF 3'UTR, other as yet unidentified destabilizing elements occur in the 5'UTR and coding region (Dibbens *et al.,* 1999), as well as elsewhere in the 3'UTR (Dibbens and Goodall, unpublished). These multiple destabilizing regions combine in their effect to produce the rapid turnover of VEGF mRNA (Dibbens *et al.,* 1999).

3.2
Stabilization of VEGFmRNA: Cis-elements and trans-acting factors

Under hypoxic conditions the VEGF mRNA half-life increases by as much as 3–4 fold (Ikeda *et al.,* 1995; Levy *et al.,* 1996b; Stein *et al.,* 1995; White *et al.,* 1995). This increase in mRNA stability involves the interplay of both stability and instability elements present in the mRNA and the trans-acting factors that interact with these elements. One approach that has been employed to identify regions that may be involved in hypoxic stabilization of the mRNA is to use the electrophoretic mobility shift assay (EMSA) to detect protein-RNA complexes that are increased in amount or affinity in extracts from hypoxic cells. Using such an approach Levy *et al.,* (1997) identified five different segments of the 3'UTR of rat VEGF that formed a hypoxia-inducible complex. The electrophoretic mobility of the complex was similar for each segment, and the different RNA segments crosscompeted for binding, suggesting that each complex has the same protein constituents. The complex was also competed for by RNA from the 3'UTR of another hypoxia-inducible mRNA, GLUT-1. No consensus sequence, beyond a general AU-richness, was evident within the five segments, which is consistent with the considerable variation in intensity of the EMSA band formed on the different segments. A single hypoxia-inducible protein of approximately 65 kDa was found to be labeled by UV-induced cross-linking to radioactive RNA probes prepared from three of the segments, and also to the GLUT-1 3'UTR (Levy *et al.,* 1996a). The identity of the 65 kDa protein remains undetermined.

The RNA-binding protein HuR has been shown to stabilize a number of mRNAs by binding to AU-rich regions of the 3'UTR (Ma *et al.,* 1997; Myer *et al.,* 1997; Rodriguez-Pascual *et al.,* 2000; Wang *et al.,* 2000a,b). The 34 kDa HuR protein was found to bind one of the five AU-rich regions of VEGF 3'UTR that was previously shown to form a hypoxia-inducible complex in the mobility shift assay (Levy *et al.,* 1997,1998). Cells expressing antisense HuR mRNA were unable to stabilize VEGF mRNA in response to hypoxia. Conversely, overexpression of HuR increased VEGF mRNA stability in normoxic conditions (Levy *et al.,* 1998). These data suggested that HuR plays an essential role in VEGF hypoxic stabilization but did not show a direct link between binding of HuR to the VEGF mRNA and stability of that mRNA. To shed further light upon this question an *in vitro* degradation assay was performed to determine if HuR directly influences VEGF mRNA stability. Using S-100 extracts and capped, polyadenylated RNA, the *in vitro* degradation of VEGF mRNA was measured. Under normoxic conditions the half life of VEGF mRNA was 8.5 minutes, compared to 30 minutes when exogenous HuR was added (Levy *et al.,* 1998). This indicated that HuR may have a direct effect on VEGF mRNA stability by binding to the 3'UTR and increasing the stability of the message. A second binding site for HuR has been identified in the rat VEGF 3'UTR (Goldberg *et al.,* 2002), although this site is not present in the mouse and human VEGF mRNAs.

The region of the rat VEGF 3'UTR designated hypoxia-inducible RNA-protein binding (HI-RPB) site I, which forms a relatively weak complex in the EMSA (Levy *et al.,* 1997), encompasses a region corresponding to a segment of the human VEGF 3'UTR that was

also found to form a complex in mobility shift assays (Claffey *et al.,* 1998). This region of the human 3'UTR conferred a two-fold hypoxic inducibility of expression on a luciferase reporter gene when inserted into the 3'UTR and was termed the hypoxia stability region (HSR). HSR RNA could be UV-cross-linked to several proteins in extracts from a melanoma cell line. One of these proteins was determined to be the hnRNP L protein (Shih and Claffey, 1999), but the identities of the other proteins, of 90 kDa, 88 kDa, 72 kDa, 56 kDa and 46 kDa, remain to be determined. The hnRNP L binding site lies within a 21 nt region that is C/A-rich. hnRNP L has been implicated in diverse functions involving interaction with other RNA-or DNA-binding proteins, including transcriptional repression (Kuninger *et al.,* 2002), internal ribosome entry site function (Hahm *et al.,* 1998) and nuclear export of mRNA (Liu and Mertz, 1995). Of special significance to its proposed role in regulating VEGF mRNA stability is the report that it may participate in the hypoxic regulation of stability of the GLUT-1 mRNA(Hamilton *et al.,* 1999).

Regulation of VEGF mRNA stability involves, in addition to elements in the 3'UTR, as yet unidentified elements in the 5'UTR and coding region. Although the VEGF 3'UTR degrades more rapidly in cell free extracts from normoxic cells than in extracts from hypoxic cells (Levy *et al.,* 1996b), in intact cells the 3'UTR is insufficient to confer hypoxic regulation of stability on a heterologous mRNA (Dibbens *et al.,* 1999; Levy *et al.,* 1997). Combinations of any two of the 5'UTR, coding region, or 3'UTR also were completely ineffective in responding to hypoxia in intact cells, whereas combining all three regions allowed recapitulation of the hypoxic stabilization seen with the endogenous VEGF mRNA (Dibbens *et al.,* 1999). Such a requirement for 5'UTR, coding region and 3'UTR for regulation of stability has also been observed with the IL-2 mRNA (Chen *et al.,* 1998). Indeed, there are some interesting parallels between the IL-2 and VEGF mRNAs. Two major RNA-binding proteins, nucleolin and the Y-box factor, YB-1, bind to the IL-2 5'UTR and are essential for the mRNA stabilization induced by T-cell activating signals. We have observed that a region of the VEGF 5'UTR that resembles the sequence of the IL-2 binding site for nucleolin and YB-1 can bind a protein complex from cell extracts that includes Y-box proteins (L.S. Coles, M.A. Bartley and G.J.Goodall, unpublished observation). The Y-box-containing cytoplasmic complex binding to the VEGF element also appears to contain another RNA-binding regulatory protein, polypyrimidine tract binding protein (PTB). A potential binding site for PTB is also present in the IL-2 element, and we have found that the Y-box/PTB-containing cytoplasmic complex that forms on the VEGF element also binds to the IL-2 element. Whether the Y-box/PTB complex is involved in regulating VEGF mRNA stability and/or translation is yet to be determined.

4.
Alternative polyadenylation sites

Two alternative polyadenylation sites are present in the mouse VEGF gene, both of which use non-canonical polyadenylation signals (Dibbens *et al.,* 2001). The use of non-canonical polyadenylation sites is usually associated with alternative or tissue-specific polyadenylation (Edwalds-Gilbert *et al.,* 1997). Usage of the upstream polyadenylation

site in the mouse VEGF gene removes two AREs (Dibbens *et al.,* 2001) and a HuR binding site (Levy *et al.,* 1998), making it likely that the stability of the shorter transcript is regulated differently than the longer transcript. However, in mouse fibroblasts the majority of transcripts were found to be polyadenylated at the downstream site, irrespective of whether the cells were cultured under normoxic or hypoxic conditions (Dibbens *et al.,* 2001). It remains to be determined whether alternative polyadenylation plays a significant regulatory role during development or in response to stimuli other than hypoxia.

5.
The 5' UTR

Most mammalian mRNAs have relatively short 5'UTRs of about 100 nt or less. However, the VEGF 5'UTR is unusually long (1039 nt in the human and 1023 in the mouse) and has a high G+C content (66% and 64% in the human and mouse, respectively). Furthermore, the human and mouse 5'UTR's are 85% identical, suggesting a functional role of the 5'UTR has been conserved throughout evolution.

5.1
Translation of the VEGF mRNA can occur by internal ribosome entry

The rate-limiting step in mRNA translation is the initial binding of the small ribosomal subunit to the mRNA. For most mRNAs this step is dependent on the cap-binding protein eIF4E, which, binds to the m^7GpppN cap structure at the 5' end of the mRNA. The eIF4E protein is present at limiting concentrations in the cell and its availability is dependent upon the phosphorylation state of its inhibitory binding partners, 4E-BP1 and 4E-BP2 (Pause *et al.,* 1994). An alternative mode of translation initiation, that does not require eIF4E and the 5' cap, involves recruitment of the translation initiation complex by an internal ribosome entry site (IRES) within the 5'UTR. Translation by internal ribosome entry was first identified with picornavirus mRNA, which does not have a 5' cap. A number of cellular mRNAs have subsequently been found to contain an IRES, particularly those that are required to be tightly regulated for normal cell function or to be translated under conditions of cellular stress.

IRESs do not have a unique or clearly defined structure, although RNA secondary structure appears to be important for IRES function. In some cases a "Y-shaped" structure within the IRES is believed to have the ability to bind either translation initiation factors or ribosomes directly (reviewed in Hellen and Sarnow, 2001). A polypyrimidine tract located from 20 to 50 nt upstream of the start codon has been shown to be absolutely required for internal initiation of some viral IRESs. This small region most likely binds a trans-acting factor that is involved either in attracting the initiation factors or in stabilizing the formation of the RNA secondary structure. Several proteins, including PTB (Hellen *et al.,* 1993), La (Holcik and Korneluk 2000), hnRNP C (Sella *et al.,* 1999), and unr (Hunt

et al., 1999), have been implicated in this process, however their mechanism of regulation is yet to be elucidated.

The VEGF 5'UTR was shown to support internal ribosome entry using assays that employ a dicistronic reporter gene (Akiri *et al.,* 1998; Huez *et al.,* 1998; Miller *et al.,* 1998; Stein *et al.,* 1998). In this assay, the putative IRES is placed between the reading frames encoding two different reporter enzymes. Because ribosomes dissociate from the mRNA after encountering a stop codon at the end of a protein coding region, the downstream reporter in a dicistronic reporter mRNA will not be translated unless an IRES is present between the two coding regions. The VEGFIRES allows translation of VEGF to be maintained during hypoxia, a stress condition that causes a reduction in overall cellular translation (Lang *et al.,* 2002; Stein *et al.,* 1998).

5.2
Location of the VEGF IRES within the 5'UTR

IRES elements have been shown to be active in only short segments of RNA, from a couple of hundred nt to just a few bases (Hellen and Sarnow, 2001). Cellular IRES elements seem to be more clearly definable than viral IRES elements; for example, the XIAP IRES has been located to approximately 30 nt (Holcik *et al.,* 1999) and the gtx IRES to just 9 (Chappell *et al.,* 2000). To determine the location of the VEGF IRES, serial deletions of the VEGF 5'UTR were made and IRES activity was determined using dual reporter assays. The mouse 5'UTR shows negligible IRES activity from nt 343 to 839, however half of the IRES activity is found from nt 474 to the start codon at nt 1023 (Miller *et al.,* 1998; Stein *et al.,* 1998). This indicates either the other half of the IRES activity comes from the first 474 nt, or the formation of secondary structure is impaired such that it only affords inefficient translation initiation. Interestingly, fusing the first 32 and the last 132 nt of the mouse 5'UTR together results in the formation of a "super" IRES, which shows 4.5-fold higher activity than the entire VEGF 5'UTR in a dicistronic assay, and 4-fold in a monocistronic assay (Stein *et al.,* 1998). These two regions show evidence of secondary structure in the "Y" shape IRES which is important for IRES function in several viral and some cellular 5'UTRs.

The human 5'UTR was found to contain two independent IRES elements by comparison of capped versus uncapped mRNAs in an *in vitro* assay (Huez *et al.,* 1998). IRES A lies at the 3' end of the 5'UTR, between nt 745 and 1038, while IRES B lies between nt 91 to 483 (Huez *et al.,* 1998). Deletion of the very 3' end of the 5'UTR (nt 955 to 1038), which is an important region for the formation of the "Y" structure, resulted in loss of more than 50% of activity (Huez *et al.,* 1998). IRES B has not been as well defined as IRES A, but nt 379–483 are essential for activity of IRES B (Huez *et al.,* 1998). How these two distinct IRESs function is not understood, but it seems likely they both function in unison to attain efficient translation when required. One function of the upstream IRES (IRES B) is to allow a CUG codon within the 5'UTR to act as an alternative translation initiation site (see below).

5.3
IRES binding proteins

The polypyrimidine tract binding protein (PTB) has been shown to be important in the function of several viral IRESs (Gosert *et al.,* 2000; reviewed in Hellen and Sarnow, 2001). Other proteins implicated in regulating IRES activity include poly r(C) binding protein, La autoantigen and UNR (Graff *et al.,* 1998; Holcik and Korneluk, 2000; Hunt *et al.,* 1999). It is thought these proteins act as translation chaperones, stabilizing the secondary structure in the 5'UTR, so that it forms a favorable template for the binding of the translation initiation factors or ribosomes directly. Two proteins thus far have been reported to bind to the 5'UTR of VEGF, one of which binds in the region of IRES A. This protein, termed plOO, has yet to be characterized, however its binding has been shown to correlate with IRES activity (Huez *et al.,* 1998). PTB has also been shown to bind to the 5'UTR, located in the first 475 nt, however IRES activity was not dependent on its binding (Huez *et al.,* 1998). PTB may be involved in another function, such as regulating the stability of the VEGF mRNA.

5.4
Utilization of alternative start codons

The major translation initiation site at the AUG codon at nt 1039 of the human VEGF transcript gives rise to protein isoforms that range in size from 121 to 206 amino acids, depending on the splicing of the alternative exons. However, an alternative site of initiation of translation has been recently identified, well upstream of the major start codon (Huez *et al.,* 2001; Meiron *et al.,* 2001; Tee and Jaffe, 2001). A CUG codon at nt 499 can act as a translation initiation codon *in vitro* and *in vivo,* producing VEGF proteins with a 180 amino acid N-terminal extension. Antibodies raised to the extension peptide detect a protein of 47 kDa in extracts prepared from human ovarian tumors and some fetal tissues (Tee and Jaffe, 2001), different mouse tissues (Huez *et al.,* 2001) and the promyelocytic cell line U937 (Meiron *et al.,* 2001), consistent with the expression of the CUGinitiated long form of VEGF in these tissues. Translation of the CUG-initiated long form of VEGF is driven by the 5' IRES (Huez *et al.,* 2001), suggesting a possible rationale for the presence of the two distinct IRESs in the VEGF 5'UTR. Minor smaller products can also arise from initiation at two other CUG codons that lie between the CUG at nt 499 and the normal AUG initiation codon (Meiron *et al.,* 2001).

The implications of the long form of VEGF are largely unknown at this stage, however there could be a link to the already well-characterized isoforms of FGF-2, which also initiates translation from CUG codons in the 5'UTR, as well as from the normal AUG initiation codon (Prats *et al.,* 1989). Like VEGF, FGF-2 is an angiogenic cytokine involved in wound healing processes. The higher molecular weight FGF-2 forms initiated by the CUG codons have within them a nuclear localization sequence, and these CUG-initiated forms lead to immortalization of bovine aortic endothelial cells and slower growth of NIH 3T3 cells (Couderc *et al.,* 1991; Quarto *et al.,* 1991). AUG-initiated forms lead to cellular transformation, indicating a different function for each isoform (Couderc *et al.,* 1991;

Quarto *et al.,* 1991). The long precursor form of VEGF was found to remain largely intracellular (Huez *et al.,* 2001) and did not stimulate the proliferation of human umbilical vein endothelial cells (Tee and Jaffe, 2001). However the long form could be proteolytically cleaved within the hydrophobic signal sequence that targets the normal form of the protein for secretion, to generate biologically active VEGF, leading to the suggestion that the long form may act as a storage form of VEGF. It will be interesting to see whether additional functions are ascribed to the long form of VEGF or to the N-terminal peptides that arise from its cleavage.

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6
Oxygen and angiogenesis

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1.
Introduction

Oxygen (O_2), the final electron acceptor in the respiratory chain of oxidative phosphorylation, is absolutely required for most living organisms, including all invertebrates and vertebrates. The delivery of atmospheric O_2 via diffusion to individual cells within a tissue is limited if the organ dimension is more than 3 mm^3 (Dachs and Tozer, 2000). Thus, in insects such as *Drosophila,* a tracheal (respiratory) system develops to meet the O_2 demand for internal tissues. Although the O_2 delivery system in mammals is far more complicated, consisting of respiratory, cardiac, vascular, and hematopoietic cells, the basic function is similar to that of the insect trachea, to transport O_2. Since these systems were developed to supply O_2, it is logical to state that the availability and concentration of O_2 regulates development of these O_2 delivery systems. Indeed, a great deal of data sustains this claim both in the *Drosophila* tracheal system and mammalian angiogenesis.

2.
O_2 sensing pathway

2.1
Adaptive response to low O_2 (hypoxia)

The body's response to hypoxia, such as rapid breathing, increased circulation, high red blood cell counts, and hemoglobin levels has long been observed. Recently, additional information concerning cellular and molecular responses to O_2 deprivation has began to accumulate. Although there is a 50–70% reduction of total RNA synthesis in hypoxic cells (Fandrey, 1995), transcription of some genes increases dramatically under low O_2. These hypoxia-responsive genes are involved in the glycolytic pathway (e.g. glucose transporter 1 and 3, phosphoglycerate kinase 1 and aldolase A), erythropoiesis (e.g. erythropoietin), angiogenesis (e.g. vascular endothelial growth factor and VEGF receptor FLT-1), vasodilatation (e.g. inducible nitric oxide synthase), and breathing rate (e.g. tyrosine

hydroxylase) (Semenza, 1998; Wenger, 2000), and function to minimize the effects caused by low O_2 both cellularly and systematically.

2.2
HIF-1 is a master regulator of hypoxia

Increased transcription rates of a select group of genes (hypoxia responsive genes) indicate involvement of a hypoxia-specific transcription factor(s). By analyzing the cis-acting DNA sequences of one hypoxia target gene, erythropoietin (EPO), a 50-bp DNA element located 3' of the human and mouse of EPO genes was identified as the hypoxia responsive element (HRE) (Wang and Semenza, 1996). Subsequently, the "Hypoxia Inducible Factor" (HIF) was purified using EPO HRE affinity chromatography on nuclear extracts from hypoxic cells (Wang and Semenza, 1995). HIF-1 was shown to be a heterodimer, consisting of HIF-1α and HIF-1β. Interestingly, HIF-1β turns out to be a previously identified protein: the aryl hydrocarbon receptor nuclear translocator (ARNT), which is involved in dioxin metabolism in conjunction with aryl hydrocarbon receptor (AHR) (Gonzalez *et al.,* 1995). Both subunits, HIF-1α and ARNT, belong to the basic helix-loop-helix (bHLH)-Period, Single-minded and ARNT (PAS) family, of mammalian proteins include AHR, CLOCK, ARNT2, and MOP3 (Bunger *et al.,* 2000; Cowden and Simon, 2002; Gekakis *et al.,* 1998; Gonzalez *et al.,* 1995; Keith *et al.,* 2001). This group of proteins has several conserved structural domains, including a bHLH region for DNA binding and dimerization, a PAS domain for target gene specificity and dimerization, and C-terminal transactivation domains. Recently, new mammalian bHLH-PAS proteins, endothelial PAS domain protein 1 (EPAS1/HRF/HLF/MOP2) (Ema *et al.,* 1997; Flamme *et al.,* 1997; Hogenesch *et al.,* 1997; Tian *et al.,* 1997) and HIF-3α have also been identified (Gu *et al.,* 1998). Both HIF-2α and HIF-3α are structurally related to HIF-1α, subject to similar protein stability regulation (see more detail below), and form dimers with ARNT. In addition, *in vitro* experiments (e.g. gel shift, reporter gene assay, ect.) indicate that both HIF-2α and HIF-3α have similar transcription activity to HIF-1α. However, our own data indicate that HIF-3α is induced by hypoxia and negatively regulates HIF-1α and HIF-2α (Hu *et al.,* unpublished data). Interestingly, "inhibitory PAS0" (iPAS), an isoform of HIF-3α, has been shown to inhibit HIF-1α and HIF-2α both in cell culture and animal models (Makino *et al.,* 2001). Besides possibly regulating different target genes, the three HIF-α subunits also exhibit quite different expression patterns, with universal expression of HIF-lα and tissue/cell specific expression for HIF-2α and HIF-3cx. Of note, HIF-2a is predominately expressed in endothelial cells and has been shown to exclusively regulate endothelial-specific genes such as Tie-2 and Flk-1 (Kappel *et al.,* 1999; Tian *et al.,* 1997). The expression of both HIF-1α and HIF-2α in endothelial cells suggests an important role for HIF in blood vessels development.

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2.3
O_2 sensing pathway

Identification of HIF-1 as a principal hypoxia response effector opens the way to address the next important question: how do cells sense O_2 deprivation, transduce O_2-mediated signals and finally activate HIF? It has been observed that hypoxia induces HIF transcription activity, mainly by stabilizing HIF-α proteins, which are rapidly turned over by ubiquitination and proteasomal degradation under normoxia (Huang et al., 1996, 1998). Subsequently, an O_2-dependent degradation (ODD) domain was identified C-terminal to the PAS domain of HIF-1α (Huang *et al.,* 1998). Renal carcinoma cells (RCC) derived from von Hippel-Lindau syndrome patients exhibit mutation in the von Hippel-Lindau protein (pVHL), high levels of HIF-α proteins, and constitutive expression of HIF target genes under normoxia (Maxwell *et al.,* 1999). Reintroduction of wild-type pVHL into RCC cells restores HIF-α's O_2-dependent instability, indicating a critical role for pVHL in HIF degradation (Maxwell *et al.,* 1999). Further biochemical studies demonstrate that pVHL is a recognition component of an E3 ubiquitin ligase for HIF degradation (Cockman *et al.,* 2000; Krek, 2000; Ohh *et al.,* 2000; Tanimoto *et al.,* 2000). pVHL-HIF interaction depends on hydroxylation of proline residues in ODD domains of HIF-α subunits (Ivan *et al.,* 2001; Jaakkola *et al.,* 2001). At least three enzymes in mammalian cells mediate this proline hydroxylation. These enzymes are members of the 2-oxoglutarate-dependent hydroxylase superfamily, whose activity requires O_2 and iron as cofactors, implying that these HIF-prolyl hydroxylases (HIF-PHs) directly sense low O_2 (Bruick and McKnight, 2001; Epstein *et al.,* 2001). In addition to HIF-α protein stability, HIF transcription activity is also regulated by O_2 levels. Abrogation of asparagine hydroxylation of the HIF-α C-teminal transactivation domain under hypoxia promotes the interaction of HIF with the transcription coactivator p300, and enhances HIF transcription activity (Lando *et al.,* 2002). Interestingly, the enzyme mediating this asparagine hydroxylation under normoxia also belongs to the 2-oxoglutaratedependent hydroxylase superfamily, whose activity is inhibited at low O_2. These results suggested a critical role of the hydroxylase superfamily in O_2 sensing. However, it is still premature to crown them as "the true O_2 sensor". Using a genetic model and a number of mitochondrial electron transporter inhibitors, we demonstrated that: 1) hypoxia increases generation of reactive O_2 species (ROS) by mitochondrial complex III; 2) ROS are necessary and sufficient in stabilizing HIF-1α and turning on HIF target genes; 3) The hypoxia response is abolished in cells depleted of mitochondrial DNA. All these results indicate that mitochondria act as the proximal O_2 sensors during hypoxia via increased production of ROS to activate HIF transcription (Chandel *et al.,* 1998,2000).

3.
O_2 and angiogenesis during normal embryogenesis

3.1
HIF function is required for vessel development

Blood vessel formation occurs via two major processes during development: "vasculogenesis" and "angiogenesis". Vasculogenesis occurs during early embryogenesis, and refers to endothelial cell differentiation and proliferation *in situ* from vascular endothelial progenitors (angioblasts) to form a primitive, immature tubular network. The first major vascular tracts, including the yolk sac and trunk axial vessels, are generated through vasculogenesis. Angiogenesis can be subdivided into angiogenic "remodeling" and angiogenic "sprouting". Angiogenic remodeling refers to a process in which the immature vessels from vasculogenesis are modified, through pruning and enlargement, to form an interconnecting network of vessels with hierarchy and mature vasculature (endothelial cells integrate tightly with supporting cells). Angiogenic sprouting refers to sprouting or enlongation of existing vessels into a previously avascular tissue. This process is responsible for vasularization of most mesodermal and ectodermal organs, including the brain and neural tube, and for most new vessel formation in the adult, including uterine cycling, placental growth, wound healing, and tumor neovascularization. It is well known that VEGF, VEGF receptors Flt-1 and Flk-1, angiopoietins, and angiopoietin receptors Tie-1 and-2 play critical roles in both vasculogenesis and angiogenesis. We believe that angiogenesis is regulated by local environmental factors such as tissue O_2 and nutrient demand. The discovery of the HIF pathway, particularly regulation of HIF activity by O_2, and HIF's function in activating angiogenic genes, established the link between these two pathways. Importantly, mutagenesis of HIF pathway genes (*Hif-1α, Hif-2a, Arnt,* and *Vhl*) in animals clearly leads to vascular defects (Gnarra *et al.,* 1997; Iyer *et al.,* 1998; Malteper *et al.,* 1997; Ryan *et al.,* 1998).

Arnt was the first gene in the HIF pathway to be disrupted (Maltepe *et al.,* 1997). There are several advantages of using *Arnt* to study the function of the HIF pathway in angiogenesis. As a common partner of all three HIF-α subunits, "knockout" of *Arnt* eliminated the function of all three HIF-α subunits. Two *Arnt*related mammalian genes, *Mop3* and *Arnt2* appear not to be involved in angiogenesis. MOP3 does not participate in hypoxia responses (Cowden and Simon, 2002), and ARNT2's hypoxia involvement is restricted in neural tissue (Keith *et al.,* 2001; Maltepe *et al.,* 2000; Michaud *et al.,* 2000). *Arnt-/-* mice exhibit embryonic lethality at E10.5 and defective angiogenesis of the yolk sac and brancial arches, indicating a critical role of ARNT in development, particularly in angiogenesis (Kozak *et al.,* 1997; Maltepe *et al.,* 1997).

Like $Arnt^{-/-}$, $Hif\text{-}1\alpha^{-/-}$ mice die at midgestation, and show a reduction in vascularization, particularly in the yolk sac and cephalic regions (Iyer *et al.,* 1998; Ryan *et al.,* 1998). The existing vessels also appear to be dilated and lacking branches, indicating that proper vasculogenesis occurred, but angiogenesis was not initiated.

Although HIF-2α is predominantly expressed in endothelial cells, $Hif\text{-}2\alpha^{-/-}$mice exhibit much milder defects in the vascular system than that of $Hif\text{-}1\alpha^{-/-}$(Peng *et al.,* 2000). In

addition, the vessel abnormalities are quite different from that of *Hif-1α*$^{-/-}$. *Hif-2α*$^{-/-}$ mice lack big vessels, probably due to failure to fuse properly or assemble into large vessels (Peng *et al.,* 2000). The lethality at day E16.5 may also be better explained as bradycardia, resulting from reduced levels of catecholamine. Catecholamine controls heart rate and is produced in the fetal organ of Zuckerkandl (OZ) in response to low O_2 levels. The high level of HIF-2a expression in the OZ indicates a critical role of HIF-2a in regulating catecholamine production (Peng *et al.,* 2000; Tian *et al.,* 1998).

As a negative regulator of HIF, mutant pVHL in tumors leads to *elevated* rates of angiogenesis. Surprisingly, *Vhl*$^{-/-}$ mice exhibit *reduced* placental vascularization (Gnarra *et al.,* 1997). This contradiction may be resolved by data from *Drosophila* studies (Adryan *et al.,* 2000). *dVHL,* an ortholog of mammalian *Vhl* in *Drosophila,* is expressed only in developing tracheal termini where branching no longer occurs, indicating that dVHL functions as an inhibitor of cell migration (Adryan *et al.,* 2000). It is well known that the pattern of primary branching is rigidly fixed and controlled by genetic programs; however, the pattern of terminal branches is variable and regulated by tissue O_2 needs (Jarecki *et al.,* 1999). Thus, a lack of pVHL would result in two completely opposite phenotypes. In primary vessel formation, lack of pVHL, leads to unregulated cell movement and failure to form vessels at all, as observed in *Vhl*$^{-/-}$ mice. On the other hand, once the primary vessels are already in place, loss of pVHL, hence uncontrolled cell migration and branching, produces elevated vasculogenesis, as noted in VHL tumors.

The previous experiments demonstrate the importance of the HIF pathway in angiogenesis during development. However, much needs to be done to better understand the function of the HIF pathway in vessel development. Multiple HIF-α (HIF-1α, HIF-2α and HIF-3α) and HIF-β (ANRT, ARNT2) subunits participate in hypoxia response. Thus, analysis of multiple mutants, such as *Hif-1α/Hif-2α* and *Arnt/Arnt2* double knockouts, as well as conditional knockout of individual genes may uncover novel roles of these factors in development and pathology.

3.2
Molecular mechanism of HIF-mediated embryonic angiogenesis

In cell culture, it is well established that HIF transcription activity is controlled by O_2 levels. Furthermore, expression of several angiogenic genes such as *Vegf, Flt-1 and Flk-1* are induced by HIF (Semenza, 2001; Semenza *et al.,* 2000). Although studies with HIF pathway knockouts suggest the presence of localized hypoxia in developing embryos, it is important to prove that hypoxia occurs in development and activates the HIF pathway. Using a hypoxia marker, pimomidazole and its antibody, immunohistochemical analysis demonstrates that hypoxia does occur as early as day E8.0, at the folding neural tube, neural mesenchymal cells, and extra-embryonic tissues such as yolk sac and allointois (Lee *et al.,* 2001). Importantly, the expression pattern of HIF-1α and its target gene, *Vegf,* were spatiotemporally identical to that of hypoxic staining (Lee *et al.,* 2001). Furthermore, platelet endothelial cell adhesion molecular (PECAM), a marker for endothelial cells, colocalizes with the hypoxia marker, indicating that endothelial cells are actively forming vessels in hypoxic regions during embryogenesis (Lee *et al.,* 2001). In

line with the above data, *Hif-1α*$^{-/-}$ embryos exhibit increased hypoxia, decreased expression of target genes *(Pgk),* and defects in the neural tube and cephalic regions where severe hypoxia was observed (Ryan *et al.,* 1998). In addition, over-expression of constitutively active HIF-1α in transgenic mice results in increased vasculization with elevated level of VEGF expression (Elson *et al.,* 2001). All these data support the idea that hypoxia stabilizes HIF, which in turn activates expression of angiogenic factors, includingVEGF, a critical growth factor for endothelial cells.

There is substantial understanding of how hypoxia regulates angiogenesis during development via the HIF pathway, however, the relative role of HIF-1α versus HIF-2α in regulating *Vegf, Flt-1, Flk-1* and *Tie-2* expression is still unclear. The differential role of HIF-lα and HIF-2a in regulating genes involved in energy metabolism may also be important in dissecting the different phenotypes observed between *Hif-1α*$^{-/-}$ and *Hif-2α*$^{-/-}$ mice. It was proposed that lethality of *Hif-1α*$^{-/-}$ mice is not the result of vascular defects, but mesenchymal cell death (Kotch *et al.,* 1999). Our own experiments indicated that HIF-1α, but not HIF-2α, induce glycolytic gene expression during hypoxia, implying that lack of energy supply in *Hif-1α*$^{-/-}$ mesenchmal cells may result in cell death (Hu *et al.,* unpublished data).

3.3
Evolution conservation between vessel development in mammal and tracheal formation in **Drosophila**

The well-studied *Drosophila* model system provides a powerful experimental tool for animal studies. The insect trachea is constructed of an epithelial monolayer of cells formed into a tubular structure to deliver O_2 to internal tissues. The pattern of the fine terminal branches in insect trachea resembles capillaries of mammalian vessels (Metzger and Krasnow, 1999). Importantly, O_2 availability determines the pattern of terminal branching of trachea as well as capillaries of mammalian vessels (Jarecki *et al.,* 1999). All these facts imply the existence of a HIF-like pathway in *Drosophila* in regulating the formation of tracheal system.

At a molecular level, *Drosophila* "Trachealess" forms a heterodimer with "Tango", their function is absolutely required in tracheal formation (Isaac and Andrew, 1996; Sonnenfeld *et al.,* 1997; Wilk *et al.,* 1996). Interestingly, Tango is a homolog to mammalian ARNT (Sonnenfeld *et al.,* 1997), while Trachealess is also a bHLH-PAS protein (Isaac and Andrew, 1996; Wilk *et al.,* 1996). However, there is no evidence so far to indicate that Trachealess protein stability is regulated by O_2 concentration. Nevertheless, hypoxia-inducible HRE-binding activity has been observed in hypoxia-treated *Drosophila* SL2 cells (Nagao *et al.,* 1996). Recently, a new *Drosophila* protein, the "similar" factor (Sima), was shown to dimerize with Tango, transactivate a HRE reporter, and accumulate in low O_2 (Bacon *et al.,* 1998; Nambu *et al.,* 1996). However, Sima does not possess the LXXLAP signature sequence in its ODD domain which is even conserved in *C. elegans* HIF-α. Furthermore, antibody against Sima did not block or supershift the HRE binding activity in SL2 hypoxic cells (Bacon *et al.,* 1998), suggesting that Sima is unlikely a major player in*Drosophila* hypoxia response. Thus, it is important to uncover other HIF homologs in

Drosophila and define their roles in tracheal development. A potential target gene for dHIF will be *Branchless,* a homolog of mammalian fibroblast growth factor (Klambt *et al.,* 1992). Branchless controls tracheal cell migration and branching pattern, and its expression is regulated by O_2 level (Jarecki *et al., 1999*; Sutherland *et al., 1996*). Besides HIF homologs, other components of a hypoxia response pathway are also conserved in *Drosophila.* dVHL has been shown to possess E3 ubiquitin ligase activity and is a critical regulator for tracheal terminal branching (Adryan *et al.,* 2000; Aso *et al.,* 2000). By RNA interference, reduced expression of *Drosophila* HIF prolyl hydroxylase results in increased expression of the HIF target gene lactate dehydrogenase under normoxia (Bruick and McKnight, 2001). Thus, an O_2 deprivation response pathway may well be conserved between *Drosophila* and mammals, and play a similar role in regulating tracheal formation in insects and angiogenesis in mammals.

4.
O_2 and tumor angiogenesis

4.1
O_2 deprivation promotes tumor angiogenesis

Angiogenesis is most prevalent during embryonic development. In adulthood, there are few normal events requiring angiogenesis. However, angiogenesis can play very important roles in the development of several diseases, such as rheumatoid arthritis, proliferative retinopathies, and solid tumors. Without new vascularization providing O_2, nutrients, and metabolic waste removal, a solid tumor usually cannot grow beyond a diameter of 3 mm^3, the distance O_2 can diffuse from a capillary vessel.

As a solid tumor grows, it suffers from multiple unfavorable growth conditions, such as glucose deprivation, hypoxia, low serum, low concentration of growth factors, and acidosis. Some of these environmental cues (e.g. acidosis and hypoxia) function as signals to induce tumor angiogenesis (Hockel and Vaupel, 2001; Xu *et al.,* 2002). Among them, hypoxia may be the most important one. During normal embryonic development, intrinsic cellular machinery responds to hypoxia and induces angiogenesis. Tumors clearly take advantage of this existing system. Although tumor angiogenesis involves complicated molecular machineries, we will only concentrate on a handful of molecules in the cellular hypoxia response pathway that is centered around the HIF transcription complex.

4.2
HIF pathway regulating tumor angiogenesis in animal models

Animal models have clearly demonstrated the essential roles played by HIF in tumor angiogenesis. As stated previously, the HIF-α subunits are stabilized in response to hypoxia and form active transcription complexes with β subunits to up-regulate an array of genes that regulate glucose and energy metabolism, iron metabolism, blood vessel formation, red blood cell production, etc. (Maxwell *et al.,* 2001). Among the large

number of HIF targets identified, the *Vegf* gene, which directly induces the proliferation of blood vessel endothelial cells, is largely responsible for hypoxia-induced angiogenesis during both normal development and tumor progression (Liu *et al.,* 1995). The astonishing results showing that loss of even one allele of the murine *Vegf* gene causes abnormal blood vessel formation and embryonic lethality underscores its pivotal role in vasculogenesis and angiogenesis (Carmeliet *et al.,* 1996; Ferrara *et al.,* 1996). Without the *Vegf* gene, ES cells cannot form teratocarcinomas in nude mice, probably due to its inability to initiate tumor angiogenesis at all (Ferrara *et al.,* 1996).

ES cells lacking the HIF-1α protein were injected into nude mice to test their ability to form teratocarcinomas (Carmeliet *et al.,* 1998; Ryan *et al.,* 1998). In both studies, compared to tumors derived from wild-type ES cells, *Hif-1*$^{-/-}$ teratocarcinomas were less vascularized. It is surprising to find that although the *Hif-(3* subunit *(Arnt)* deficient embryo also dies of aberrant angiogenesis (Maltepe *et al., 1997), Arnt*$^{-/-}$ ES gave rise to well-vascularized teratocarcinomas undistinguishable from tumors derived from wild-type cells (Maltepe *et al.,* 2000). However, closer examination of these tumors revealed that they contain large amounts of neuronal tissues in which the ARNT homolog, ARNT-2, expression is very high (Maltepe *et al.,* 2000). On the other hand, when introduced into nude mice, hepatoma cells expressing neither the ARNT nor ARNT-2 proteins exhibited reduced tumor vascularity (Maxwell *et al.,* 1997). These studies suggest that there are functional redundancies between these two β subunits. In response to the hypoxia stress in tumors, either one of them would be necessary and sufficient to form transcriptional active heterodimer with a subunits to launch an efficient angiogenesis response.

It is interesting to note that transgenic mice overexpressing VEGF in keratinocytes develop overgrowth of a leaky vasculature, reminiscent of the tortuous and leaky blood vessels in tumors (Larcher *et al.,* 1998). However, overexpression of a constitutively active HIF-1α protein in the same kind of cell exclusively causes increased growth of well-organized blood vessel networks, phenotypically undistinguishable from normal vessels except in density (Elson *et al.,* 2001). The discrepancy of blood vessel formation between HIF overexpressed in normal tissue versus HIF overexpressed in tumors suggests that HIF controls the expression of other genes cooperating with VEGF to insure well-organized vessel growth. It is conceivable that in normal tissues, when the layout of existing developmental signals interacting with the HIF pathway is orderly, the growth of new vessels is well organized. On the other hand, because tumors lack the orderly distribution of these developmental signals, HIF overexpression in tumors results in a chaotic vessel network.

pVHL functions as the E3 ubiquitin ligase of HIF-α subunits to keep their protein levels very low under normoxic conditions (Cockman *et al.,* 2000; Maxwell *et al.,* 1999; Mole *et al.,* 2001; Ohh *et al.,* 2000; Tanimoto *et al.,* 2000). As expected, lack of functional pVHL leads to activation of the HIF pathway even without hypoxia (Wiesener *et al.,* 2001). Deletion of the *Vhl* gene in mouse livers causes steatosis, increased vascularization, and blood-filled cavities (Haase *et al.,* 2001). The expression of several downstream HIF targets such as *Vegf, Glut-1* and *Epo* is up-regulated in *Vhl*$^{-/-}$livers (Haase *et al.,* 2001). This study confirms that pVHL is a negative regulator of HIF-induced angiogenesis, and strongly suggests that vascularized tumors contain deregulated HIF activity.

4.3
Frequent deregulation of HIF pathway in human malignancies

Mutation and LOH (Loss of Heterozygosity) of the *VHL* gene account for the occurrence of early onset of multiple tumors such as hemangioblastomas, CCRCs (clear cell renal carcinomas) and pheochromocytomas in patients with VHL syndrome (Kondo and Kaelin, 2001). Lack of functional pVHL also occurs in the majority of sporadic hemangioblastomas of the CNS and sporadic CRCCs (Kondo and Kaelin, 2001). A common characteristic of these tumors without functional pVHL is that they are extremely vascularized. This is consistent with the notion that pVHL negatively regulates the expression of HIF-α subunits, whose over-expression leads to angiogenesis. Indeed, both HIF-α proteins and their downstream targets such as *VEGF* are overexpressed in $VHL^{-/-}$ renal carcinoma cells (Maxwell *et al.,* 1999). Such deregulation can be corrected via reintroducing pVHL into these cells (Maxwell *et al.,* 1999).

Besides loss of functional pVHL, clinical studies have revealed that deregulation of other points in the HIF pathway are frequent in human tumors. Under normal conditions, HIF-α proteins are expressed at levels lower than the detectable threshold (Zhong *et al.,* 1999). On the contrary, easily detectable HIF-1α and HIF-2α protein expression even in tumors with functional pVHL is frequently observed (Zhong *et al.,* 1999). Since the majority of solid tumors suffer from chronic hypoxia, it is conceivable that such hypoxic conditions cause the upregulation of HIF-α proteins. However, whether hypoxia is the only mechanism leading to these pVHL-independent HIF levels increases is not very clear. There is evidence suggesting that loss of several tumor suppressor proteins results in elevated levels of HIF-α subunits. In several glioblastoma cell lines, inactivation of the tumor suppressor PTEN leads to HIF-1α protein stabilization, presumably through activation of the growth promoting PI(3)K-Akt pathway (Zundel *et al.,* 2000). However, the involvement of Akt in upregulating HIF expression and activity has been challenged recently (Arsham *et al.,* 2002). Physical association of HIF-1α protein with the tumor suppressor p53 has also been reported (An *et al.,* 1998; Ravi *et al.,* 2000). In one of these studies, it was suggested that this association leads to the degradation HIF-1α protein via indirect attachment to the p53 ubiquiting ligase MDM2. Therefore, loss of p53 can contribute to the HIF-1α stabilization (Ravi *et al.,* 2000). One other tumor suppressor, $p14^{ARF}$, whose expression is also frequently lost in human malignancies, inhibits HIF activity by sequestering HIF-1α protein to the nucleolus (Fatyol and Szalay, 2001). Therefore, tumors lacking $p14^{ARF}$ may exhibit increased HIF activity. It is interesting to note that in certain RCC cell lines with no functional pVHL, HIF-1α protein expression is also lost (Maxwell *et al.,* 1999). This suggests that if there is no other transcriptionally active a subunit unidentified, HIF-2α subunits alone can associate with β subunits and cause sufficient angiogenesis in tumor.

The most direct regulator of vessel endothelial cell proliferation is VEGF. As expected, results derived from studies of a wide range of human tumors show a vast majority overexpress VEGF protein (Ferrara and Davis-Smyth, 1997). Furthermore, the expression of *VEGF* in tumors has been correlated with the degree of tumor vascularity. High VEGF expression level appears to overlap with hypoxic subregions in tumors (Plate

et al., 1992; Shweiki *et al.,* 1992). The *VEGF* promoter contains a Hypoxia Response Element (HRE) and *VEGF* is a *bona fide* transcription target of HIF (Minchenko *et al.,* 1994). Additional effects of hypoxia on the level of *VEGF* expression have been reported. For instance, hypoxia enhances both the stability of *VEGF* mRNA and the translation of *VEGF* (Akiri *et al.*, 1998; Damert *et al.,* 1997; Levy *et al.,* 1996,1997; Shima *et al.,* 1995; Stein *et al.,* 1998). Besides HIF, deregulation of other growth control pathways can lead to VEGF overexpression. The accumulation of adenosine and activation of c-Src may be involved in hypoxia-induced *VEGF* stimulation (Mukhopadhyay *et al.,* 1995; Takagi *et al.,* 1996). Finally, *VEGF* can be induced by cytokines such as EFG, TGF-β, TGF-α and IL-1 (Frank *et al.,* 1995; Goldman *et al.,* 1993; Li *et al.,* 1995; Pertovaara *et al.,* 1994). In addition to cytokines, certain differentiation and transforming events also seem to upregulate *VEGF* expression (Claffey *et al.,* 1992; Garrido *et al.,* 1993). As probably the most potent angiogenic factor in tumors, VEGF is presently one of the main targets for tumor anti-angiogenesis therapy.

Although the HIF pathway is frequently deregulated in human tumors, with the exception of VHL patients, up to now, there have been no examples of genetic alteration directly affecting other molecules in the HIF pathway. It is possible that in addition to HIF overexpression, loss of pVHL leads to tumorigenesis due to HIF-independent functions of pVHL, such as its ability to regulate cell-matrix interactions (Ivan and Kaelin, 2001). Loss of this function may offer selective growth advantage only in certain cell types. If true, it explains why VHL patients develop only certain kinds of tumors such as CCRC and CNS hemangioblastomas, although loss of pVHL should promote angiogenesis in all kinds of tissues. Since it is very likely that individual HIF-α proteins regulate cell growth and metabolism differently, it seems possible that VHL mutant tumors only occur in certain organs because HIF expression in these cells is intrinsically different.

Under certain circumstances, events promoting tumor angiogenesis may not result in optimal growth of individual cells. This may be the case for hypoxiainduced HIF-1α expression. HIF-1α strongly induces genes involved in glycolysis (Seagroves *et al.,* 2001), which lead to more rapid accumulation of acidosis under hypoxic conditions and consequent cellular apoptosis. Therefore, the disadvantage of HIF-1α expression in causing acidosis may compete with its benefit in providing nutrients and energy. Moreover, some studies suggest that HIF-1α expression leads to p53 stabilization, resulting in growth inhibition to tumors harboring wild-type p53 (An *et al.,* 1998). There are conflicting results in terms of tumor growth derived from studies using *Hif-1α*$^{-/-}$ ES cells to generate teratocarcinomas. In one study, tumors lacking HIF-1α protein showed stunted growth after 3 weeks due to increased apoptosis (Ryan *et al.,* 1998). In contrast, another study found that lack of HIF-1 expression allows tumors to grow at an accelerated rate, presumably due to decreased hypoxia-induced apoptosis (Carmeliet *et al.,* 1998). In some RCC cell lines lacking pVHL expression, HIF-1α expression is not detectable (Maxwell *et al.,* 1999). This raises the possibility that high levels of HIF-1α expression are somehow selected against during tumor development. All these results may reflect the dual role on cell survival and proliferation played by the HIF-1α protein in response to hypoxic stress and the outcome may depend on the specific microenvironment and the genetic background.

As a solid tumor grows, insufficient blood supply does not only cause hypoxia but also nutrient and growth factor deprivation and acidosis. It is possible that cells may sense these stresses and induce angiogenesis as well. Compared to hypoxia, how and whether cells respond to nutrient deprivation and acidosis with an angiogenic response is a largely unexplored area. Although not confirmed, several studies suggest that the HIF pathway can respond to these environmental changes. *VEGF* expression may be induced by glucose deprivation independent of HIF activity (Kotch *et al.,* 1999). Glucose deprivation has been reported to induce HIF complex activation (Maltepe *et al.,* 1997). However, this result may be due to the mild increase in hypoxia during culture instead of glucose deprivation (Brain Keith, unpublished observations). Multiple genes in the glycolytic pathway are transcription targets of HIF (Maxwell *et al.,* 2001). When the glycolytic pathway is stimulated during hypoxia, the cellular microenvironment will become acidic, leading to elevated VEGF expression independent of hypoxia (Xu *et al.,* 2002). Lack of HIF-1α expression has been shown to either protect (Brusselmans *et al.,* 2001) or sensitize (Williams *et al.,* 2002) cells to hypoglycemia-induced apoptosis. *Hif-2*$^{-/-}$ cells may also have a growth advantage over wild-type cells in a hypoglycemic environment (Brusselmans *et al.,* 2001). Obviously, more work is needed to further elucidate the molecular mechanisms of cellular responses to these microenvironment changes and their role played in angiogenesis.

5. Conclusions

In conclusion, HIF is the center of the cellular O_2-sensing machinery and plays a crucial role in tumor angiogenesis and sustaining tumor development. This is because hypoxia is a naturally occurring stress in solid tumors during their progression. In order to overcome this growth-limiting condition, tumors employ the existing hypoxia response pathway to induce angiogenesis. Moreover, taking advantage of other deregulated growth control pathways or genetic defects, tumors may utilize HIF to cause angiogenesis even without hypoxia. It is also possible that the HIF pathway is used for sensing other microenvironmental changes caused by decreased profusion, leading to tumor angiogenesis. New molecules, such as the HIF proline hydroxylase (Bruick and McKnight, 2001; Epstein *et al.,* 2001), HIF asparagine hydroxylase (Lando *et al.,* 2002) and HIF-3α (Gu *et al.,* 1998), have significant functions in regulating the cellular hypoxia response and have been just recently characterized. It is very likely that tumors achieve angiogenesis by interfering with the expression and activities of these molecules as well. One exciting new area will be to explore the deregulation of these molecules in tumors and their potential role in tumor angiogenesis.

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7
Angiogenesis during zebrafish development

Suk-Won Jin, Benno Jungblut and Didier Y.R.Stainier

1. Introduction

The zebrafish model system was developed in part to enable unbiased large-scale genetic screens in a vertebrate organism. Such screens allow the discovery of genes required in defined developmental processes and the subsequent analyses of their biological function. The genetic dissection of zebrafish vascular development is facilitated by the properties of the zebrafish embryo, as well as an ever-increasing set of tools available to analyze the nature and function of the genes, including a detailed description of vascular development in the zebrafish embryo.

Similar to other vertebrates, vascular assembly in zebrafish occurs by two distinct mechanisms: vasculogenesis and angiogenesis. Vasculogenesis is a process in which endothelial cell precursors, called angioblasts, differentiate and assemble into primitive blood vessels. Vasculogenesis can be further divided into two types (Coffin and Poole, 1991). Type I refers to the *in situ* differentiation of angioblasts to form new vessels, while type II refers to the process in which angioblasts from pre-existing blood vessels migrate and form new vessels. Angiogenesis is a process in which a new vessel is formed by either sprouting from, or splitting of, pre-existing vessels. Previous studies suggest that at least some aspects of both vasculogenesis and angiogenesis are genetically programmed processes in zebrafish (Childs *et al.,* 2002; Isogai *et al.,* 2001; Lawson *et al.,* 2001), although local environmental cues such as oxygen concentration also appear to play a critical role, especially during angiogenesis (Giordano and Johnson, 2001; Shweiki *et al.,* 1992).

In this chapter, we will first review why the zebrafish is a powerful yet underutilized model system to study vascular development. We will then review vascular development in zebrafish, discussing the various molecules as they are utilized temporally in this process.

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2.
Zebrafish as a model organism to study vascular development

Zebrafish has several distinctive attributes, which make it an attractive model system to study vascular development *(Figure 1)*. First of all, its externally fertilized and transparent embryos allow relatively easy manipulation and imaging during early development. Moreover, rapid embryonic development and small body size allow zebrafish embryos to survive and develop relatively normally in the absence of a functional cardiovascular system up to 5 days post fertilization (dpf) (Stainier, 2001). This feature allows the easy identification and recovery of mutations affecting vascular development. The large number of progeny and relatively fast life cycle allows conventional chemical mutagenesis schemes that were successfully used in invertebrates, notably in *C. elegans* and *Drosophila*

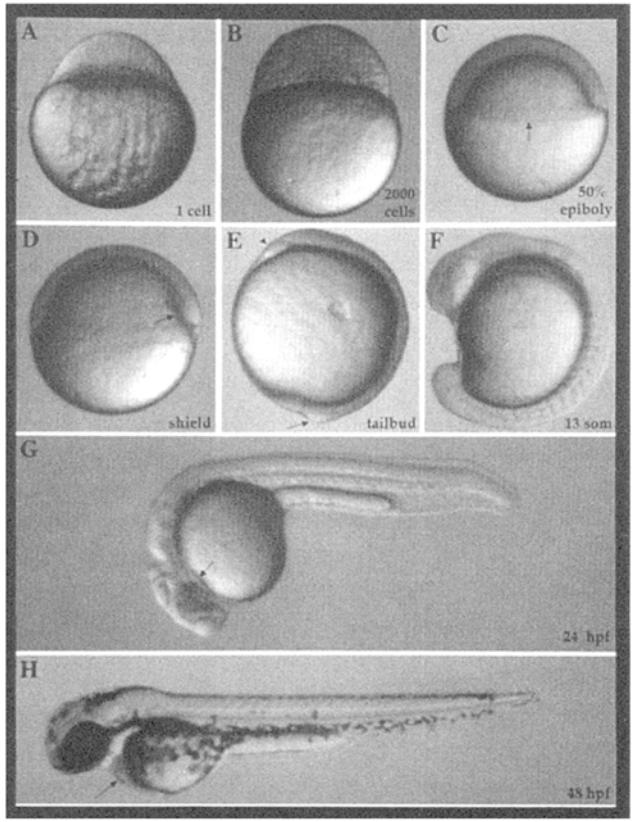

Figure 1. Zebrafish development. A. Fertilized egg (1 cell stage). B. 2000 cell stage. C. 50% epiboly. D. Shield stage. E. Tailbud. F. 13-somite stage. G. 24 hpf. The heart (arrow) starts beating and major axial vessels such as the dorsal aorta and posterior cardinal vein are starting to be used in circulation. H. 48 hpf. Circulation extends to secondary vessels such as the intersegmental vessels and sub-intestinal vein.

(Brenner, 1974; Niisslein-Volhard and Wieschaus, 1980). This approach allows the identification and recovery of mutations that affect vascular development in a non-biased manner. Indeed, initial large-scale mutagenesis screens identified more than one hundred mutations affecting various aspects of cardiovascular development, a partial list of which is shown in *Table 1* (Chen *et al.,* 1996; Stainie *et al.,* 1996).

An increasing genomic infrastructure facilitates the subsequent positional cloning of the genes affected by these mutations. Several genes regulating vascular development have been cloned and further analyzed. For example, *gridlock,* which was first identified in the Boston screen (Weinstein *et al.,* 1995) was recently cloned and shown to encode a Hairy Related Transcription factor (HRT), which acts downstream of Notch signaling (Zhong *et al.,* 2000). Recent advances in antisense oligonucleotides based gene knock-down technology such as morpholinos allows a prompt analysis of gene function by a "reverse genetics" approach (Nasevicius and Ekker, 2000; Nasevicius *et al.,* 2000).

Various imaging techniques available in zebrafish greatly enhance the functional analyses of genes being studied. Two complementary imaging techniques are widely used to dissect the *in vivo* function of a specific gene: Green Fluorescent Protein (GFP) labeling

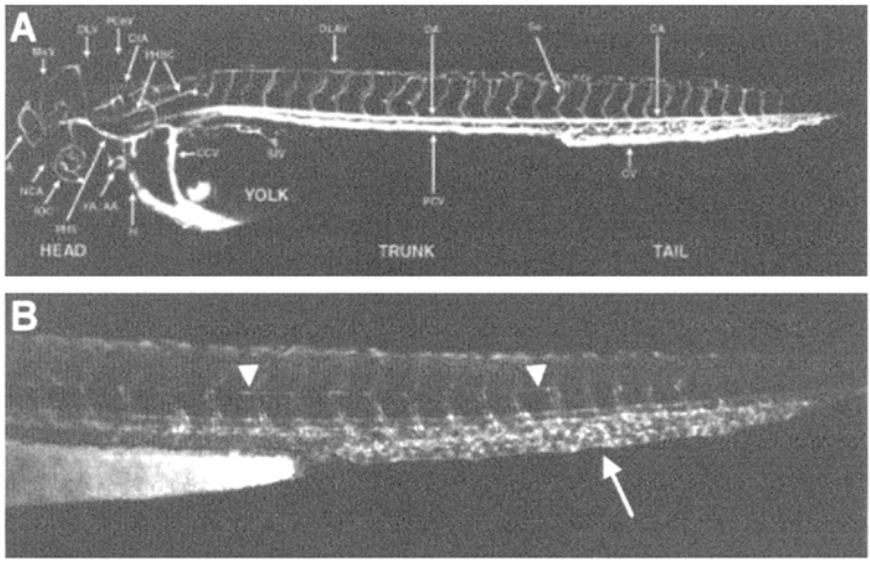

Figure 2. Molecular tools to study vascular development. A. Microangiography of threeday-old zebrafish embryo. Most of the major vessels are already formed. Notice that the posterior cardinal vein (CV) remains as a plexus at this stage, which will undergo remodeling and eventually form one defined posterior cardinal vein. Only perfused vessels are visualized (Isogai et al., 2001). B. Confocal micrograph of a live *tie2*::GFP transgenic zebrafish embryo. Endothelial cells of a three-day-old embryo are visualized by GFP expression. Perfused vessels can be observed in addition to cellular extensions not visible by microangiography (white arrowheads). The white arrow shows the posterior cardinal vein plexus, and white arrowheads show the sprouting of the parachordal vessels from intersegmental vessels.

Table 1. Cardiovascular mutants in zebrafish (partial list)

Mutation	Protein affected	Phenotype	Reference
cloche	Unknown	No endothelial cells	Stainier *et al.*, 1995
gridlock	Hairy related bHLH protein		Weinstein *et al.*, 1995; Zhong *et al.*, 2000
kurzschluss	Unknown	No trunk circulation	Chen *et al.*, 1996
violet beauregarde	Activin receptor-like tyrosine kinase-1		Roman *et al,* in press
out of bounds	Unknown	Misguided vessel growth	Childs *et al.*, 2002
mindbomb	Unknown		Lawson *et al.*, 2001
heart of glass	Unknown		Stainier *et al.*, 1995
santa	Unknown	Enlarged heart	Stainier *et al.*, 1995
valentine	Unknown		Stainier *et al.*, 1996
bonnie and clyde	Mixer related homeodomain protein		Kupperman *et al.*, 2000; Stainier *et al.*, 1996
casanova	SOX related protein		Chen *et al.*, 1996; Kikuchi *et al.*, 2000
faust	Gata5		Chen *et al.*, 1996; Kikuchi *et al.*, 2001
miles apart	Sphingosine 1-phosphate receptor	Cardia bifida	Chen *et al.*, 1996; Reiter *et al.*, 1999
natter	Fibronectin		Jiang *et al.*, 1996; Trinh *et al.*, submitted
two of heart	Unknown		Alexander *et al.*, 1998
cardiofunk	Unknown	No valve formation	Stainier *et al.*, 1996; Walsh and Stainier, 2001
jekyll	UDP-glucose dehydrogenase		Alexander *et al.*, 1998
pickwick	Titin		Xu *et al.*, 2002
silent heart	Cardiac Troponin T		Chen *et al.*, 1996; Sehnert *et al.*, 2002
still heart	Unknown	No heart beat	Chen *et al.*, 1996
viper	Unknown		Chen *et al.*, 1996

via transgenic fish and microangiography (*Figure 2*). The relative ease of generating transgenic zebrafish by microinjection facilitates the use of transgenic zebrafish to study vascular development. Several transgenic lines, useful for cardiovascular studies, are available (Motoike *et al.,* 2000). For example, *tie-2*::GFP transgenic zebrafish, which express GFP in developing endothelial cells, has been used to study defects in vascular development (Motoike *et al.,* 2000; Walsh and Stainier, 2001). Another transgenic zebrafish, which harbors a *fli-1*::EGFP transgene has been recently developed (Brant Weinstein, personal communication; Roman *et al.,* in press). These transgenic zebrafish with endothelial cell-specific GFP expression assist the analyses of mutations affecting initial vessel formation, although it is difficult to determine whether specific vessels are functional, since GFP expression is only indicating the presence of differentiated endothelial cells. Microangiography offers an excellent alternative to transgenic zebrafish in analyzing circulation patterns. Briefly, fluorophorecontaining beads are injected to either the Sinus Venosus or the Posterior Cardinal Vein of the zebrafish embryo, then analyzed by confocal microscopy. The image acquired from microangiography shows only perfused vessels actively participating in circulation. Thus, subtle defects in vascular development such as abnormal remodeling can be detected (Childs *et al.,* 2002; Weinstein *et al.,* 1995). And many mutations that affect later aspects of vascular development have been analyzed in depth by microangiography.

3.
Vascular development in zebrafish

Initial vasculogenesis in zebrafish is generally analogous to that in other vertebrate systems. In chick and mouse embryos, angioblasts differentiate from the lateral plate mesoderm to form a pair of axial vessels such as the dorsal aorta and axial vein which are positioned laterally during early development. In zebrafish, angioblasts originate from cells located throughout the marginal zone of the gastrulating embryo, as shown by lineage analysis carried out at the onset of gastrulation (Warga and Niisslein-Volhard, 1999). At the 12-somite stage, angioblasts are found in an area of the lateral plate mesoderm often referred to as the Intermediate Cell Mass (ICM).

Compared to other vertebrates, two unique aspects of vascular development have been noted in zebrafish. First of all, the angioblasts migrate to the midline where they form the dorsal aorta and posterior cardinal vein instead of forming pairs of more laterally located vessels as in other vertebrates. In addition, initial vasculogenesis occurs quickly in zebrafish without the intermediate stages found in other vertebrates (Isogai *et al.,* 2001).

3.1
Initial specification of angioblasts

Hemangioblasts and cloche. The close association of the developing endothelial and hematopoietic lineages has led to the hypothesis that these two lineages share common precursors, termed hemangioblasts (Risau and Flamme, 1995). Several observations support the existence of the hemangioblasts. First of all, single embryonic stem cells that

express *flk-1,* also known as Vascular Endothelial Growth Factor Receptor 2 (VEGFR-2), have the potential to differentiate into angioblasts or hematopoietic precursors in vitro (Nishikawa et al., 1998). In addition, *flk-1* knock-out mice lack functional vasculature and have a severe reduction of blood cells (Shalaby et al., 1995). Furthermore, overexpression of *scl/Tal-1* in mouse increases the number of both endothelial and hematopoietic cells. Taken together, these data suggest that there are common precursors for both cell types, which express *flk-1* and *scl/Tal-1* Lineage analyses in zebrafish also support the existence of hemangioblasts on the ventral side of the blastula although more detailed studies will be required to understand in detail these lineage relationships.

Another line of support for the existence of hemangioblasts comes from the zebrafish mutant, *cloche* (Stainier *et al.,* 1995). Homozygous *cloche* mutants show no blood vessels or endocardium, and the number of red blood cell precursors is severely reduced (Liao *et al.,* 1997,1998; Parker and Stainier, 1999; Stainier *et al., 1995*): *flk 1* positive cells are eliminated except for a small patch of cells located in the posterior region of the embryo. These *flk-1*-positive cells fail to express later endothelial cell markers, such as *tie1* and *tie2.* Furthermore, the expression of many other hematopoietic and endothelial cell markers such as *gata1, scl/Tal-1,* and *fli1* are severely reduced or absent in *cloche* mutant embryos (Liao *et al.,* 1997, 1998). These data suggest that *cloche* is required for both endothelial cell differentiation and blood cell formation (Stainier *et al.,* 1995).

The expression patterns of hematopoietic and endothelial markers in *cloche* mutants indicate that *cloche* might be the earliest known modulator of endothelial cell differentiation (Liao *et al.,* 1997). However, the gene affected by the *cloche* mutation has yet to be identified. Determining the molecular identity of *cloche* will enhance our understanding of how the hemangioblast cell lineage is determined and differentiates to form a functional vasculature. Furthermore, it may provide an opportunity to explore novel methods of treating various human conditions affecting vascular formation.

Vascular endothelial growth factors and their receptors. The differentiation of angioblasts seems to require the activities of receptor tyrosine kinases, such as the vascular endothelial growth factor (VEGF) receptors, *flt-1* (VEGFR-1) and *flk-1* (VEGFR-2) (Veikkola and Alitalo, 1999). VEGF ligands trigger the growth of blood vessels in culture, and targeted gene inactivation of different VEGF genes in mouse has shown that they are essential for endothelial cell differentiation (Veikkola and Alitalo, 1999). Similarly, inactivation of the VEGFR genes in mouse results in varying degrees of endothelial cell differentiation defects, suggesting that the activity of these genes is critical for proper vascular development (Dumont *et al.,* 1995; Fong *et al.,* 1995). The VEGF signaling pathway also plays a critical role during vascular development in zebrafish. Two members of the VEGF family have been identified in zebrafish to date, *vegfa* and *vegfc* (Liang *et al.,* 1998; Olofsson *et al.,* unpublished data). The expression of *vegfa* starts as early as 80% epiboly and persists until 72 hpf. At the 18-somite stage, *vegfa* expression gets restricted to bilateral regions. By 24 hpf, most of the early expression of *vegfa* fades away, except in the pronephric glomeruli (Liang *et al.,* 2001). Furthermore, gene knock-down studies using morpholino anti-sense oligonucleotides show that VEGF function is essential for proper vascular development (Nasevicius *et al.,* 2000).

Two VEGFRs have been identified in zebrafish to date, *flk 1* and *flt4.* The expression of *flk1* can be detected starting at the 5-somite stage in two lateral stripes of cells located in the lateral plate mesoderm (Liao *et al.,* 1997, 1998; Thompson *et al.,* 1998). The flkl-positive cell population extends both anteriorly and posteriorly as the embryo develops. At the 18-somite stage, a cluster of *flk1*positive cells arises medially and is thought to give rise to the endocardium *(Figure 3A)* (Liao *et al.,* 1997). All endothelial cells appear to express flkl at 24 hpf though the expression level in arterial endothelial cells seems to be higher than that in venous endothelial cells *(Figure 3B)* and expression continues within sprouting endothelial cells until later stages. The other VEGFR, *flt4,* starts to be expressed by the seven somite stage (Thompson *et al.,* 1998). Its expression pattern largely overlaps with that of *flk1*, although it is more strongly expressed in the developing axial vein than in the dorsal aorta, and is restricted to venous endothelial cells by the 30-somite stage (Lawson *et al.,* 2001). The expression *of flt4* cannot be detected by 48 hpf, suggesting that it plays an early role in the developing vasculature (Thompson *et al.,* 1998).

Endothelial cell-specific transcription factors. At least three transcription factor genes are known to be required for endothelial cell differentiation after the initial formation of the angioblasts: *scl/Tal-1, fli1,* and *hhex* (Brown *et al.,* 2000; Drake *et al.,* 1997). A basic helix-loop-helix (bHLH) transcription factor gene, *scl/Tal-1,* is also expressed widely in both angioblasts and hematopoietic precursors during development. Its expression can be detected as early as the first somite stage in the lateral plate mesoderm, and becomes stronger in the ICM at the 5-somite stage (Liao *et al.,* 1998). As is the case in mouse, the overexpression of the *scl/Tal-1* ortholog in zebrafish increases the number of endothelial and blood cells (Gering *et al.,* 1998; Liao *et al.,* 1998). An ETS family transcription factor gene,*fli1*, is expressed starting at around the 5-somite stage in zebrafish. It is expressed in both arterial and venous endothelial cells at similar levels (Brown *et al.,* 2000; Lawson *et al.,* 2001).

The homeobox gene *hhex* also appears to be involved in endothelial cell differentiation. It is expressed at 5-somite stage in the lateral plate mesoderm, and is expressed as two lateral stripes later on. Its expression diminishes after the onset of circulation (Liao *et al.,* 2000b). Although the overexpression of *hhex* can induce the expression of other endothelial cell markers such as *flk1* and *fli1*, a deletion allele of *hhex* does not show any obvious vascular defects, suggesting that the activity of *hhex* is not essential for angioblast formation. It is possible that the activity of *scl/Tal-1* can compensate for the loss of *hhex* activity since they appear to activate the expression of each other (Liao *et al.,* 2000b).

3.2
Vasculogenesis

Arterial-venous differentiation. Until recently, it has been thought that the differences between arterial and venous endothelial cells do not arise until after the onset of circulation, as a result of the response to hemodynamic forces. However, recent reports suggest that the differences between these two endothelial cell types occur long before the onset of circulation in zebrafish (Lawson *et al.,* 2001; Zhong *et al.,* 2001). It is not well

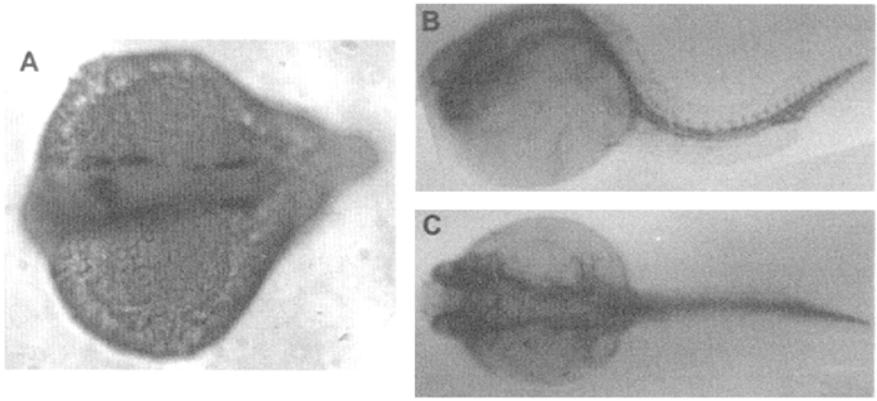

Figure 3. Expression pattern of endothelial cell markers, *flk-1*. A. Wild-type 18-somite stage embryo stained with *flk1*. At the 18-somite stage,*flk-1*-positive cells are found near the midline around the heart field. These cells appear to contribute to the endocardium later. Two lateral stripes of *flk1*-positive cells will form axial arteries as well as some cranial vessels such as the first aortic arch. B. Wild-type 24 hpf embryo, lateral view. Most of the vascular structures express *flk*1 at 24 hpf including the intersegmental vessels, which sprout from the DA and PCV. C. Wild-type 24 hpf embryo, dorsal view.

understood exactly how nascent angioblasts differentiate into either arterial or venous endothelial cells. However, it appears that both arterial endothelial cells and venous endothelial cells arise from a similar region of the ICM (Zhong *et al.,* 2001).

The function of a recently cloned gene, *gridlock,* appears to promote arterial cell fate after the initial differentiation of angioblasts (Zhong *et al.,* 2001). Homozygous *gridlock* mutants display no circulation in the trunk region due to a block formed at the anterior end of the dorsal aorta, where the two anterior lateral dorsal aortae fuse to form a single midline dorsal aorta. However, most of the vascular structure in the head region and anterior trunk appears to be normal.

Positional cloning of *gridlock* revealed that it encodes a member of the Hairy/Enhancer-of-split related family of basic helix-loop-helix (bHLH) proteins, a transcriptional repressor associated with the Notch signaling pathway (Zhong *et al.,* 2000). It is expressed as early as the 10-somite stage in the lateral plate mesoderm, and is restricted to the dorsal aorta by the 30-somite stage. This expression pattern suggests that *gridlock* function is required early on during angioblast differentiation (Zhong *et al.,* 2000).

Hairy-related bHLH transcription factors are known to "single out" precursor cells from equivalence "groups" during cell fate determination. Therefore it is possible that Gridlock functions as a molecular cue to define arterial angioblasts from a group of bipotential angioblasts that can differentiate into either arterial or venous angioblasts. It is not clear exactly how Gridlock controls arterial versus venous fates of developing angioblasts. Zhong and colleagues suggest that Gridlock regulates angioblast cell fate determination by suppressing venous fates via *ephrin-B2/EphB4* signalling (Zhong *et al.,* 2001). This idea is supported by gene knock-down studies with morpholino anti-sense

oligonucleotides targeting *gridlock* (Zhong *et al.,* 2001). The morpholino-injected embryos show an expanded posterior cardinal vein at the expense of the dorsal aorta. However, overexpression of *gridlock* does not cause expansion of the dorsal aorta, suggesting that *gridlock* function is necessary but not sufficient for the formation of the dorsal aorta.

A function of *ephrin-B2/EphB4* signaling in blood vessel development has been shown in mice: the targeted knock-out of *Ephrin-B2,* a transmembrane ligand gene which is preferentially expressed in arterial endothelial cells, causes severely reduced branching of cranial capillaries and defects in the remodeling of extraembryonic vessels (Wang *et al.,* 1998). Mice lacking *EphB4,* an *Ephrin-B2* receptor that is expressed in venous endothelial cells, exhibit a similar phenotype (Adams *et al.,* 1999; Gerety *et al.,* 1999; Wang et al., 1998). In zebrafish, *ephrin-B2* is initially expressed around the 16-somite stage, when the migrating angioblasts reach the midline. Like murine *Ephrin-B2,* zebrafish *ephrin-B2* expression is also restricted to arterial endothelial cells (Lawson *et al.,* 2001). Two putative zebrafish orthologs of *EphB4, rtk5* and *rtk8,* have been isolated (Cooke *et al.,* 2001). The expression pattern of *rtk5* has been reported to be restricted to venous endothelial cells, which is reminiscent of murine *EphB4* (Lawson *et al.,* 2001).

The role of surrounding structures in vasculogenesis. The notochord appears to play a critical role in the formation of the dorsal aorta (Fouquet *et al.,* 1997; Mullins *et al.,* 1996; Odenthal *et al.,* 1996; Talbot *et al.,* 1995; van Eeden *et al.,* 1996), as well as that of several other midline tissues including the floor plate and ventral neural tube. Dorsal aorta formation is perturbed in mutants such as *no tail* and *floating head,* which completely lack the notochord. In these mutants, circulation appears to be normal in the anterior trunk and in the head region of the embryo, while circulation in the posterior trunk is severely limited or eliminated. The expression patterns of *flk1* in these mutants suggests that angioblasts are present, but highly disorganized (Fouquet *et al.,* 1997; Sumoy *et al.,* 1997). *floating head* mutants also lack the hypochord, a transient structure that consists of a single row of cells and is located immediately ventral to the notochord and dorsal to the dorsal aorta (Eriksson and Lofberg, 2000). The hypochord appears to be important for the formation of the dorsal aorta in both zebrafish and Xenopus. In zebrafish, angioblasts aggregate ventral to the hypochord and form the axial vessels between the 15-and 16-somite stages (Eriksson and Lofberg, 2000). The dorsal aorta is thereby intimately associated with the hypochord. In *Xenopus,* the notochord is required to induce the differentiation of the hypochord from the endoderm. Hypochord cells express a soluble isoform of VEGF, which has been shown to be chemotactic for angioblasts *in vivo* (Cleaver and Krieg, 1998; Cleaver *et al.,* 2000).

Many mutations affecting different aspects of developmental processes, such as somite or notochord formation, display abnormal vasculogenesis in addition to their primary defects (Chen *et al.,* 1996; van Eeden *et al.,* 1996). Several mutations that affect the formation of the horizontal myoseptum show potential vasculogenesis defects in the trunk region. For example *you-too,* a mutation in the *gli2* gene, affects the formation of the horizontal myoseptum, and the differentiation of muscle pioneer cells and effectively eliminates circulation in the trunk area, possibly due to a malformation of the dorsal aorta. A similar phenotype has been observed in *sonic you/shh* and *chameleon,* both of which also affect the horizontal myoseptum (Chen *et al.,* 1996; van Eeden *et al.,* 1996). Recently

hedgehog expression in the notochord has been hypothesized to activate VEGF expression in the somites, which in turn triggers the activation of Notch signaling to promote dorsal aorta formation (Brant Weinstein, personal communication).

Angiopoietins and Tie-1/Tie-2 *receptors.* Two receptor tyrosine kinase genes, the orphan receptor gene *tie-1* and the Angiopoietin-1 receptor gene *tie-2,* are expressed at later stages of endothelial cell differentiation in mouse, and are required for the integrity and survival of endothelial cells (Puri *et al.,* 1995; Sato *et al.,* 1995). In zebrafish, both *tie1* and *tie2* expression are first detected by the 18somite stage, and their expression patterns largely overlap with that of *flk1.* They are universally expressed in the developing vasculature including the dorsal aorta, posterior cardinal vein, intersegmental vessels, and endocardium (Liao *et al.,* 1997; Lyons *et al.,* 1998).

To date four ligands of the Tie2 receptor have been identified. The best characterized of these ligands are angiopoietins (Ang) 1 and 2 (Davis *et al.,* 1996; Maisonpierre *et al.,* 1997). The zebrafish orthologs of Angiopoietin-1 and Angiopoietin-2 have recently been cloned and their expression pattern analyzed (Pham *et al.,* 2001). Both genes are expressed in regions close to sites of active blood vessel formation. Although Ang1 and Ang2 are thought to compete for binding to the Tie2 receptor, the spatial expression domains of these genes do not overlap considerably in zebrafish. Both *ang1* and *ang2* are expressed in ventral head mesenchyme, however while *ang1* is expressed in the hypochord and the ventromedial regions of the somites, *ang2* is expressed only in the anterior trunk and the pronephros.

Ang1 has been shown to be chemotactic *in vitro* (Witzenbichler *et al.,* 1998), therefore it could play an instructive role in directing angioblast migration to the position of the dorsal aorta. Persisting Ang1 expression in the hypochord could also be important for the maturation and stabilization of the dorsal aorta by promoting endothelial cell survival (Kwak *et al.,* 1999). The expression patterns of *ang1* and *ang2* seem to be unaffected in *cloche* mutants indicating that the presence of vascular endothelial cells is not essential for the regulation of *ang1* and *ang2* expression (Pham *et al.,* 2001).

3.3
Angiogenesis

Although the analysis of the molecular mechanisms of angiogenesis in zebrafish is only emerging, several recent studies suggest that the zebrafish will provide insight into this process. First of all, Isogai and colleagues recently described the vascular development in wild-type zebrafish embryos up to 7dpf, which provides an essential reference for the mutants to be compared to (Isogai *et al.,* 2001). Secondly, several mutations affecting angiogenesis in zebrafish have been reported (Childs *et al.,* 2002; Roman *et al.,* in press; Weinstein *et al.,* 1995). For example, two mutations, *mindbomb* and *out of bounds,* affect the formation of intersegmental vessels. Other mutations such as *violet beauregarde* and *kurzschluss,* affect only specific cranial vessels. These mutations, combined with on-going screens for additional mutations affecting angiogenesis, will provide an opportunity to delineate the molecular mechanism underlying angiogenesis.

Vascufogenesis type II vs. angiogenesis. After the initial vasculogenesis forms the major vessels and circulation is established, the second wave of vessel formation begins either through vasculogenesis type II or angiogenesis. At least some of the secondary vessels are formed by vasculogenesis type II. For example, intersegmental vessels in zebrafish appear to form via sprouting of angioblasts from either the dorsal aorta. Childs and colleagues described the formation of intersegmental vessels via vasculogenesis type II in detail (Childs *et al.,* 2002). Briefly, they observed that single angioblasts from the dorsal aorta migrate to form the intersegmental vessels. Later, angioblasts in the dorsal aorta near the intersegmental vessels form a cellular extension dorsally to generate a T-shaped structure. These angioblasts will connect the angioblasts in the intersegmental vessels to the major trunk vessels.

Most of the vessels that provide blood flow to internal organs such as hepatic portal vessel, which supplies blood flow to liver, and sub-intestinal vein, which transports nutrient from the yolk to the embryo, form via angiogenesis and remodeling (Childs *et al.,* 2002; Isogai *et al.,* 2001). For example, the sub-intestinal vein undergoes morphological and topological changes during development before it is finally located underneath the intestinal bulb (Isogai *et al.,* 2001).

Several observations suggest that vessel formation during zebrafish development is genetically programmed, in spite of its highly dynamic nature. For example, at least the first four sets of intersegmental vessels show a pre-determined pattern of alternating connection to dorsal aorta and posterior cardinal vein (Isogai *et al.,* 2001). In addition, formation of the posterior cardinal vein through remodeling of the posterior cardinal venous plexus occurs invariantly. By 7 days post fertilization, the vasculature matures and does not appear to change further. Below we will discuss several genes that have been reported to act during the formation of specific angiogenic vessels in zebrafish, especially the intersegmental vessels.

mindbomb. The *mindbomb* mutation affects the sprouting of intersegmental vessels. The severe neurogenic phenotype and other defects suggest that *mindbomb* encodes a component of the Notch pathway (Jiang *et al.,* 1996). Aberrant intersegmental vessel projection and defects in dorsal aorta formation have been reported in *mindbomb* mutants (Lawson *et al.,* 2001). For example, the intersegmental vessels in *mindbomb* mutants show ectopic sproutings which invade somites. However, angioblast markers such as *flk1, fli1,* and *tie1,* appear to be expressed normally. Furthermore, the expression of the arterial endothelial cell marker, *gridlock,* is not affected by the *mindbomb* mutation (Lawson et al., 2001). These observations suggest that the vascular defects caused by *mindbomb* do not affect the initial differentiation or migration of angioblasts, but later aspects of vascular development.

Out of bounds. Another mutant affecting intersegmental vessel projection, *out of bounds,* has been recently reported (Childs et al., 2002). At the 17-somite stage, *out of bounds* mutants show precocious sprouting of intersegmental vessels from the dorsal aorta, which does not occur until the 24-somite stage in wild-type. Furthermore, these precociously formed intersegmental vessels are misguided: many of them display a tortuous path, and occasionally, even loop back to the dorsal aorta. In addition, intersegmental vessels sprout

at abnormal sites and invade the neighboring somites. It appears that *out of bounds* affects ventral aspects of intersegmental vessel formation more severely than dorsal aspects.

Homozygous *out of bounds* mutant embryos injected with *vegf* morpholinos show the same patterning defects as non-injected homozygous mutant embryos, although the precocious migration defects are suppressed by *vegf* morpholinos. This observation suggests that the intersegmental vessel defects in *out of bounds* mutants do not appear to be caused by precocious sprouting, but rather by misguided migration. The molecular nature of the *out of bounds* gene remains unknown: however, transplantation experiments suggest that the defects caused by the *out of bounds* mutation are cell non-autonomous (Childs *et al.,* 2002).

violet beauregarde and kurzschluss. Several other zebrafish mutations that affect other angiogenic vessels have been identified in zebrafish. Two mutations, *kurzschluss* and *violet beauregarde,* appear to affect brain vasculature specifically. Caudal circulation is missing in these mutants. The molecular nature of *kurzschluss* still remains to be discovered. However, *violet beauregarde* has recently been cloned and shown to encode an activin receptor-like tyrosine kinase-1, also known as Alk-1 (Roman *et al.,* in press). Homozygous *violet beauregarde* mutants display a rather normal circulation at early stages. By 48 hpf, most of the blood cells are restricted to dilated cranial vessels and progressively the head becomes edematous. However, vessel patterning is not affected by the *violet beauregarde* mutation, although there are differences in vessel caliber and potency. It appears that the angiogenic defects in *violet beauregarde* are due to the excessive proliferation of angioblasts in the brain region, not to a failure to remodel.

Although its transcripts can be detected as early as tailbud stage by RT-PCR, *violet beauregarde/alk1* expression cannot be detected until 40 hpf by *in situ* hybridization. The expression pattern of *violet beauregarde* largely overlaps with that of *tie1*. While strongest expression can be observed in the first aortic arch, and carotid artery, which are dilated in mutants, it is expressed in most of the developing vessels including the dorsal aorta and posterior cardinal vein.

4.
Angiogenesis as a stress response and its potential use

Like many other vertebrates, zebrafish also display angiogenesis in response to aberrant environmental conditions, such as exposure to hypoxic conditions. Although zebrafish embryos can develop relatively normally without a functional cardiovascular system during the first 5 days, starting at day 3, embryos lacking blood circulation show increasing developmental defects. These defects are most likely caused by an impaired supply of oxygen. The mutation in cardiac troponin T, *silent heart,* provides an attractive system to explore the hypoxic response of zebrafish. Although *silent heart* mutant embryos do not display any circulation due to the lack of heartbeat, their vasculature develops relatively normal up to 60 hpf (Sehnert *et al.,* 2002). However, these embryos display ectopic angiogenesis in later development. Endogenous alkaline phosphatase activity staining shows that *silent heart* mutant embryos develop ectopic outgrowth of angiogenic vessels from the sub-intestinal vein on the yolk *(Figure 4)*. Although homozygous *silent*

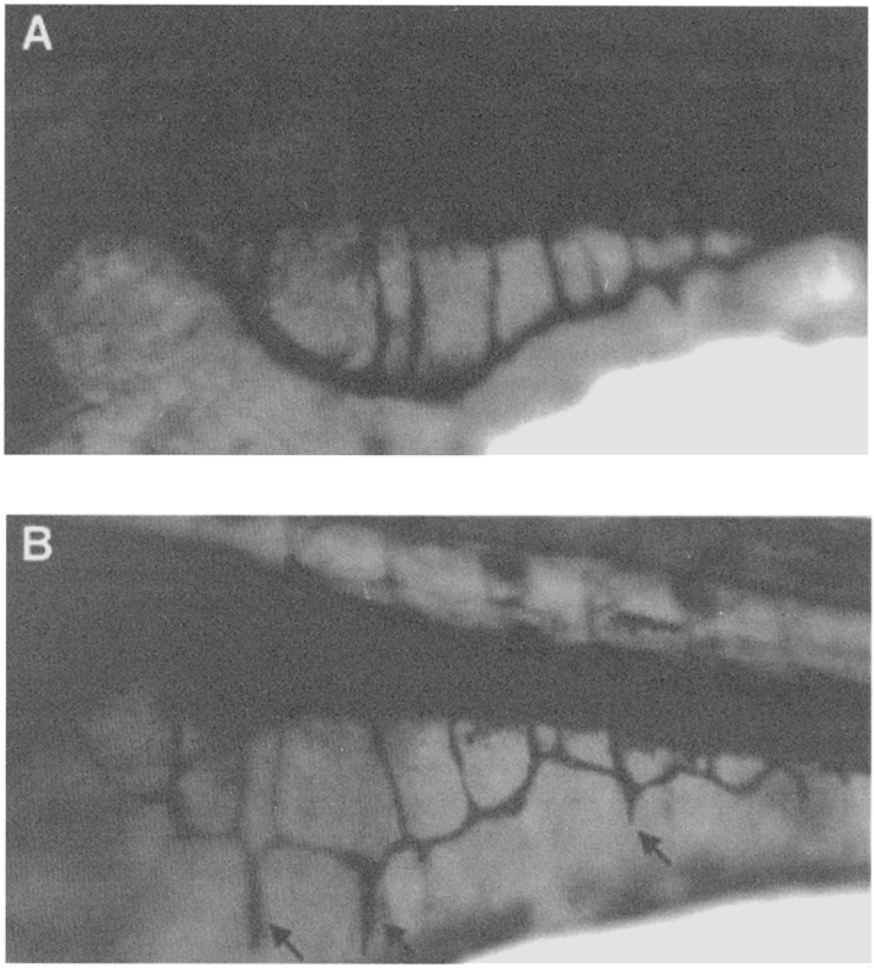

Figure 4. Hypoxic response in zebrafish. A: Wild-type embryo at 72 hpf stained for endogenous alkaline phosphatase activity. The subintestinal vein is formed without any additional sprouting. B. Homozygous *silent heart* mutant at 72 hpf stained for endogenous alkaline phosphatase activity. The subintestinal vein is formed, but distorted. Furthermore, ectopic sproutings from the sub-intestinal vein are clearly evident (black arrows).

heart mutant embryos do not display any obvious ectopic sprouting of intersegmental vessels, it is not clear whether other secondary vessels formed by vasculogenesis type II are affected by the hypoxic response. These data suggest that the vasculature of developing zebrafish embryos do respond to hypoxic stress.

This observation demonstrates that the sub-intestinal vessel in zebrafish visualized by staining for endogenous alkaline phosphatase activity can provide an effective *in vivo* assay system for drug discovery. Currently, cell culture assays are widely used to search for

novel anti-angiogenic molecules. However, complex responses to these potential drugs in multicellular organisms such as adverse side effects are hard to identify in cell cultures. Zebrafish can provide an alternative and complementary model system to cell culture. Due to their small size and relative ease of culture, zebrafish embryos can be raised in large numbers. In addition, many chemicals dissolved in DMSO can be readily delivered to zebrafish embryos cultured in multi-well plates by simple diffusion. It has been shown recently that small chemicals known to affect cardiovascular development in other systems also function in zebrafish embryo (Peterson *et al.,* 2000), supporting the employment of zebrafish as an assay system for novel drug discovery for human vascular conditions.

5. Summary and perspectives

Recent studies of vascular development in zebrafish have enhanced our knowledge of early vascular development. As mentioned earlier, zebrafish provide a new model system to study vascular development, complementary to other vertebrate systems. Several on-going large-scale genetic screens in a number of labs, including our own, will help us delineate molecular mechanisms underlying vasculogenesis and angiogenesis. In addition, the sequencing of the zebrafish genome as well as emerging technologies such as morpholino-based gene knockdown and trangenesis make the zebrafish an increasingly powerful model system.

The information acquired from studying zebrafish vascular development can be directly applied to other vertebrate systems. Many genes implicated in vascular abnormalities in human have orthologous genes in zebrafish, and these specific genes also affect cardiovascular development in zebrafish *(Table 2)*. In addition, the zebrafish appears to be a promising system to screen for small molecules regulating angiogenesis thereby expanding the use of this model system. The next 5–10 years should see the rapid expansion of the contributions of this system to our undersanding of vascular development.

Table 2. Zebrafish model for human diseases

Zebrafish mutation	Gene affected	Human disease	Reference
dracula	ferrochelatase	Erythropoietic protoporphyria	Childs *et al.,* 2000
gridlock	bHLH transcription factor	Coarctation of the aorta	Weinstein *et al.,* 1995; Zhong *et al.,* 2000
one-eyed pinhead	EGF-CFC factor	Holoprosencephaly	Schier *et al.,* 1997; Zhang *et al.,* 1998
pickwick	titin	Cardiomyopathy	Xu *et al.,* 2002
riesling	β-spectrin	Hereditary spherocytosis	Liao *et al.,* 2000a
sauternes	δ-aminolevulinate synthease	Congenital sideroblastic Anemia	Brownlie *et al.,* 1998
silent heart	cardiac troponin T	Cardiomyopathy	Sehnert *et al.,* 2002
violet beauregarde	activin receptor-like kinase 1	Hereditary hemorrhagic telangiectasia	Roman *et al.,* in press
zinfandel	globin	Thalassemia type disorder	Chan *et al.,* 1997

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8
Skin transgenic models of angiogenesis

Gavin Thurston and Nicholas W.Gale

1. Introduction

Angiogenesis is a complex process involving multiple cell types and growth factors. As putative pro-and anti-angiogenic factors are identified in various model systems, there is a need to validate these factors, and extend their biology, in more complex systems. Transgenic mice have proven a powerful tool for studying the basic mechanisms of angiogenesis and examining the role of angiogenesis in various diseases. In most transgenic approaches, a growth factor or intracellular signaling protein is overexpressed under the control of a cell-or tissue-specific promoter. If expressed in a constitutive system, the factor will not produce an immunologic reaction and can act throughout development. If expressed in an inducible system, the factor can be switched on and act on otherwise normal tissue. This ability—to match the expression pattern of the gene to the experimental question—is crucial to dissecting the actions of angiogenic factors.

One particularly useful transgenic system has been to create mice which overexpress secreted proteins under skin-specific promoters. The technical foundation for skin-specific transgenic mice was initiated by the characterization of several skin-specific promoters. These promoters have since been used to express various secreted proteins that can act in a paracrine fashion on one or more of the non-epithelial cell types in the skin or in an autocrine fashion on adjacent epithelial cells. Alternatively, cytosolic or nuclear proteins can be expressed that act in an autocrine fashion on the same epithelial cells that produce them. The factors driven by skin-specific promoters may have direct angiogenic effects on endothelial cells, or may have indirect effects by acting via cells in the interstitium or epithelium, or by recruiting inflammatory cells.

The skin transgenic system has several advantages. First, there are several wellcharacterized and widely available promoters which can efficiently direct expression of the transgene to skin and a few other sites of epidermis. Second, mice can tolerate fairly large perturbations to the skin and its vasculature. Third, the skin is relatively easy to study, because of its ease of access, as well as the availability of various models of inflammation and cancer (see below). Indeed, because of its experimental accessibility, the skin vasculature has been the site examined in several lines of gene-deleted mice (eg. TSP2 (Lange-Asschenfeldt *et al.,* 2002); Angiopoietin-2 (N.Gale *et al.,* 2002).

In many cases, proteins secreted by the epidermal cells are confined to the dermis and epidermis, and can not be detected systemically (i.e., in serum). However, some transgenically overexpressed proteins are found at high levels in serum and can exert significant distal actions. In general, this distinction seems to depend on the nature of the secreted protein. Proteins with matrix-binding sites and/or poor pharmacokinetics (e.g., VEGF, Angiopoetin-1), are not likely to be found in serum. In contrast, proteins with weak matrix-binding properties and good pharmacokinetics, such as many circulating protein hormones (e.g., leptin (Larcher *et al.,* 2001)) or immunoglobin Fc fusion proteins (e.g., VEGF-R3-Ig (Makinen *et al.,* 2001)), can be found at effective levels in serum.

In this chapter, we outline the use of the skin as a model tissue, review the different molecular genetic approaches used to generate skin transgenic mice, describe the types of vascular responses observed, and discuss the implications for our understanding of the process of angiogenesis.

2. Structure and vasculature of mouse skin

The mouse skin consists of epidermal and dermal compartments *(Figure 1)*. The epidermis, a multi-layered epithelium sitting on a basement membrane, is the cellular target of all skin-specific promoters used to generate transgenic mice to date. Basal epithelial cells can proliferate and act as stem cells, while differentiated cells move upward through the layers, become keratinized, and are finally shed. In some regions of mouse skin, such as the tongue, the epidermis and dermis interdigitate, forming structures known as papillae, whereas in other regions of skin, such as the ear skin, the two layers are normally flat. The dermis is composed of loose connective tissue, fibroblasts, and fat cells, as well as many specialized cells and structures such as immune surveillance cells, glands, hair follicles, and structures for sensation of touch. The dermis also contains the blood and lymphatic vasculatures.

The blood vasculature differs in different regions of skin, and also differs somewhat between mice and humans. In general, the skin blood vessels enter from the deeper layers of connective tissue and, upon entering the dermis, form a deep plexus and another, more superficial plexus just beneath the epidermis. In regions of mouse skin with dermal papillae, such as the tongue, each papilla is supplied by a capillary loop emanating from the vascular plexus at the base of the papillae *(Figure 1)*. In regions of skin without papillae, the superficial plexus is less complex, without capillary loops. Each hair follicle, and associated sebaceous gland, is also supplied by its own capillary loop or small vascular network. Following the general pattern of the blood vessels, the lymphatic vessels are associated with the two plexus layers and extend into the dermal papillae (Skobe and Detmar, 2000).

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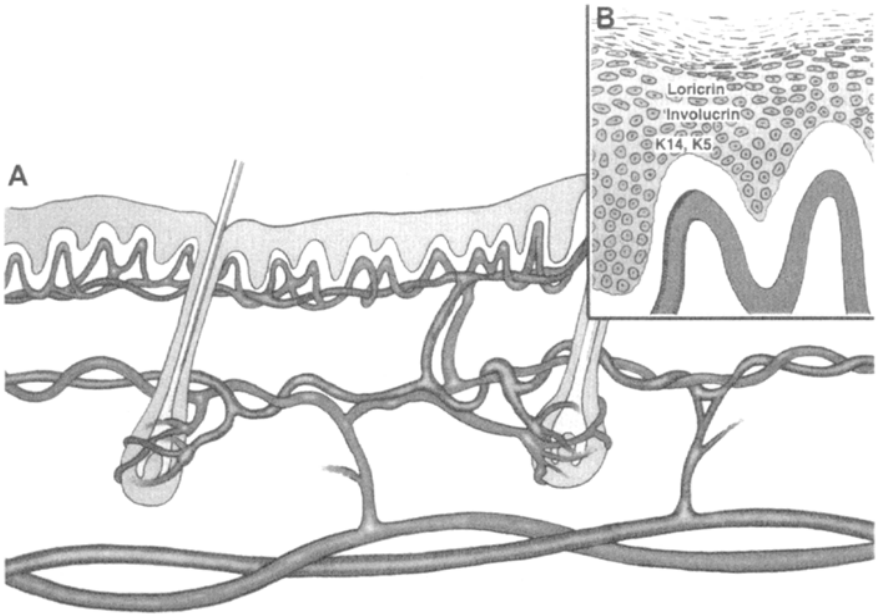

Figure 1. Diagram of histologic features of skin. (A) Blood vessels in skin, with deeper network and network beneath epidermis, in addition to capillary loops in dermal papillae and near hair follicles. (B) Detail of epidermis, showing site of expression of various trangene promoters.

The skin vasculature is not static (Peus and Pittelkow, 1996). Significant evolution of the blood and lymphatic vessels occurs during the postnatal period. In general, the superficial dermal blood vessels are more numerous in neonatal mice, and are pruned and differentiated in the first few weeks of life. In addition, the blood vessels of adult mice remodel in conjunction with the cycle of the hair follicle (Peus and Pittelkow, 1996; Yano *et al.,* 2001).

3. Approaches to study blood vessels in mouse skin

Skin blood vessels have been examined in histologic cross sections, often by using immunostaining to highlight endothelial cells, basement membrane, or vascular smooth muscle cells. Various antibodies to the endothelial marker PECAM-1 (CD31) are useful in frozen and paraffin sections. More recently, techniques have been developed to examine skin vessels in skin whole mounts (Thurston *et al.,* 1999, 2000). The ear skin is particularly useful because of its relatively thin dermis. The complete network of dermal blood and lymphatic vessels can be exposed by peeling the ear open and dissecting the thin cartilage and muscle layers. The vessels in the ear skin can be stained by intravascular perfusion of labeled lectins (Elson *et al.,* 2000; Thurston *et al.,* 1996, 1998), by

immersion in vascular-specific antibodies (Thurston *et al.,* 1999), or by LacZ staining of appropriate transgenic or gene knock-in mice (Veikkola *et al.,* 2001).

Acute and chronic inflammation can be readily induced and examined in skin. An approach commonly referred to as the Miles assay has been adapted to study vascular leakage in mouse skin. This approach involves intravenous injection of a tracer dye, followed by local application or injection of an inflammatory agent. In guinea pigs and rats, the assay is commonly used on the dorsal and flank skin, but our experience has been that the ear skin of mice provides a much more consistent response than the back skin. Using Evans blue as a tracer dye, we have measured leakage induced by local application of acute inflammatory mediators (Thurston *et al.,* 1999, 2000). Chronic leakage in certain skin transgenic mice has also been measured using tracer dyes (Thurston *et al.,* 1999). Uptake of extravascular fluid via dermal lymphatics can also be assessed by dermal injection of tracer dye and subsequent visualization of lymphatic vessels (Veikkola *et al.,* 2001).

Other types of inflammation can also be readily induced in mouse skin, including delayed type hypersensitivity (DTH) reaction (Lange-Asschenfeldt *et* al., 2002; Wille *et al.,* 1998), allergic reactions (Ma *et al.,* 2002), contact sensitivity (Enk and Katz, 1995), irritant contact dermatitis (Sunderkotter *et al.,* 2001), and localized Shwartzman reaction (Pepys *et al.,* 1982; Subramaniam *et al.,* 1996; Sunderkotter *et al.,* 2001). In addition, skin carcinogenesis can be induced by a variety of methods. Several skin transgenic mice exhibit consistent skin tumorigenesis and angiogenesis (D' Angelo *et al.,* 2000; Ishikawa *et al.,* 2001; and see below), and hyperplasia and/or carcinogenesis can be induced by prolonged topical application of TPA (Feith *et al.,* 2001; Gonzalez-Suarez *et al.,* 2001), UV radiation (Bickers and Athar, 2000), and various other agents (Tennant *et al.,* 1996). Thus in addition to being an important target organ in its own right, the skin is an excellent model system to study various diseases with angiogenic or vascular remodeling components.

4.
Molecular approaches to skin transgenics

A variety of molecular genetic approaches has been exploited for skin-specific expression of transgenes in mice. Currently, transgene expression systems can be broadly divided into two categories, constitutive (monogenic) and inducible (bigenic) systems. A simple monogenic system which may also be useful for inducible expression in skin is based on the naturally inducible K6 gene (see below). The K6 system may be a viable compromise for some investigations. Each system has potential advantages and disadvantages, but all involve introducing recombinant DNA into the mouse genome.

Several different transduction methods have been used to introduce recombinant DNA and generate transgenic mice, including pronuclear injection of oocytes with DNA constructs, adenovirus-or retrovirus-mediated transduction of oocytes, or electroporation of DNA constructs into embryonic stem cells which are then incorporated into host embryos to generate chimeric mice. All of these methods result in random integration of the DNA constructs into the host genome, and, with the exceptions of ES cell electroporation and viral gene delivery, result in variable numbers of integrations.

Experience has shown that expression levels of the transgene often do not correlate with the number of integrated copies.

The site of integration of the transgene into the host genome can have at least two important consequences. First, it has been estimated that 30% of transgenes integrate into other genes, frequently disrupting their expression, and at times leading to phenotypes which are the result of disruption of the bystander gene rather than gain of the transgene (Miao *et al.,* 1994). Second, transgene expression levels and even the fidelity of tissue-specific expression are highly dependent on the site of integration.

The site of integration of the transgene is difficult to characterize in mice generated using the above-mentioned transduction methods. Minimally, large numbers of founder mice must be screened to identify those with the correct location and level of expression of the transgene. However, it is worth noting that many of the recently generated mouse lines for the bigenic strategies described below have already been characterized.

Some recently developed systems can produce mice in which a prescribed locus in the genome is used as the site of transgene integration. The advantages of such an approach include the necessity of characterizing far fewer founder animals. Examples of such prescribed sites for integration are ROSA26 (Soriano, 1999; Zambrowicz *et al.,* 1997) and BT5 loci (Michael *et al.,* 1999). These loci contain genes which are normally very broadly expressed, and transgenes incorporated at these loci appear to be similarly broadly expressed. Disadvantages of this method are that more consideration needs to go into the design of the targeting constructs, which are more complex than most standard transgene constructs, and the longer time needed to produce F1 mice from germline-transmitting chimeric males.

In a variation on this approach, loci which give the desired pattern of gene expression can be used as the site of integration. This approach is similar to a knock-in strategy, and may result in disruption of the gene at the integration site. However, disruption of a single allele is unlikely to perturb organ development. Another advantage of this approach is that the copy number of the transgene can be controlled. To date, inducer lines of mice have not yet been produced in which the common transactivating components are expressed in the epidermis through replacement of endogenous genes.

4.1
Constitutive skin expression systems

Constitutive expression systems have several advantages over the more complicated, inducible expression systems. These advantages generally stem from the relative simplicity and speed with which a model can be established, and are largely attributable to the fact that the constitutive expression systems are monogenic. However, constitutive expression systems obviously have less spatial and temporal control of gene induction.

The best characterized systems for skin-specific transgenics use the promoters of various epidermal structural proteins to drive the transgene. Such promoters are expressed in specific layers of stratified epithelium, and include those of keratins, involucrin, and loricrin. Because a number of skin-specific promoters have been characterized, transgene expression can be reliably targeted to specific layers of the epidermis.

In many organ systems, constitutive over-expression of growth factors or other signaling molecules has deleterious effects on the development of the mouse. An investigator attempting to generate such transgenic models must typically screen a large number of founders to identify lines in which the transgene expresses at low levels. In comparison, expression of transgenes in the skin is seldom lethal. Although the skin is developmentally required for the formation of limbs and other appendages, it appears to be otherwise dispensable for prenatal development. In a testament to the dispensability of the skin, mice deficient for p63 develop rather normally but are born without skin (Mills *et al.,* 1999; Yang *et al.,* 1999). The skin is, however, essential to maintain hydration after birth. Thus, the skin is a relatively good system to study transgenic signaling molecules with relatively limited concerns about embryonic lethality.

Here we will review some of the better characterized promoter systems for expressing transgenes in epidermis. We include a summary of what is known about timing and patterns of expression, and attempt to provide information about the particular regions of the promoters, which appear to be critical for getting appropriate expression in the target tissue.

Keratin 14 (K14) and Keratin 5 (K5) promoters. The K14 and K5 promoters are the most commonly used promoter systems for driving expression of transgenes in the skin. The timing, location and extent of expression by these two promoters appear to be very similar (Byrne *et al.,* 1994). Using specific antisera as well as marker-gene expressing transgenic mice, K14 and K5 are expressed as early as embryonic day 9.5. Expression of K5 and K14 appears at the bi-layered epithelial cell stage (Byrne *et al.,* 1994), where it is limited to the inner embryonic basal layer of skin (Kopan and Fuchs, 1989). By E10.5, expression is directed to first branchial arch structures as well as broadening in expression in embryonic ectoderm. A dramatic increase in K5 promoter activity occurs between E13. 5 and E14.5, particularly in regions where hair follicles and whiskers are developing. By E16.5 promoter activity can be observed over the entire embryo surface. In adults, K5 and K14 are expressed in most multilayered epithelium, where their expression is limited to the basal layer of proliferating keratinocytes in contact with the basement membrane (Ramirez *et al.,* 1994). In addition to stratified squamous epithelium such as the skin, expression can be observed in vibrissa and hair follicles and oral epithelium, palate, tongue, esophagus, and stomach. The promoters are strongly active in dividing cells of epidermis and some other stratified squamous epithelia (Ramirez *et al.,* 1994; Vasioukhin *et al.,* 1999).

In transgenic mice, K5 and K14 promoters direct expression of transgenes to the basal layer of stratified epithelium, and notably, to the keratinocyte stem cells resident in this layer. Upon differentiation of basal cells, expression of K14 and K5 are downregulated, and thus these promoters are not active in suprabasal keratinocytes. Constructs containing approximately 2.5 kb of 5' upstream and approximately 700 bp of 3' downstream sequence of the human K14 gene were used to drive expression of transgenes, and produced expression patterns similar to the engogenous K14 gene (Vasioukhin *et al.,* 1999; Vassar *et al.,* 1989). Studies with the K5 promoter generally use approximately 5.3 kb of 5' upstream sequence derived from the bovine K5 gene (Indra *et al.,* 1999; Ramirez *et al.,* 1994).

Involucrin promoter. Involucrin is a marker of keratinocyte terminal differentiation. In adults involucrin is not expressed in the basal layers of stratified squamous epithelium, but rather is specifically expressed in the suprabasal layers *(Figure 1)*. Involucrin expression is observed in the suprabasal layers of the epidermis, vagina, cervix, trachea, esophagus and conjunctiva. Transgenic mouse lines show that a 3.7 kb (Carroll *et al.,* 1993) or 2.5 kb (Crish *et al.,* 1993) upstream regulatory region from the human involucrin gene are capable of directing tissue-, differentiation-and layer-specific expression of transgenes in mice. In contrast transgenes containing less than approximately 2 kb of upstream sequence do not appear to be capable of driving specific expression of transgenes (Crish *et al.,* 1993). Transgenic mice employing the involucrin promoter have not been described for angiogenic factors, however the promoter should be useful in systems in which expression in the suprabasal as opposed to the basal epidermal cell layers is desired. It is worth noting that this promoter may be less optimal than K5 or K14 promoters for driving the expression of Cre or FLP for the purpose of modifying expression of target genes in skin, and it will not target epidermal stem cells, but only cells committed to epidermal terminal differentiation.

Loricrin promoter. Loricrin is normally expressed in the cornified cell envelope of skin cells. However, transgenic mice using parts of the loricrin promoter can express transgenes in all layers of the epidermis (DiSepio *et al.,* 1999). DiSepio *et al.,* evaluated the expression of 6.5-kb and 14-kb loricrin promoter constructs in transgenic mice. Significantly, expression was found in all layers of the epidermis of the 6.5-kb transgenics, including basal and spinous cells. The expression of the 14-kb version, however, was indistinguishable from that of the endogenous gene, confirming that the additional sequences contain negative regulatory elements that restrict loricrin expression to the granular layer in vivo.

4.2
Inducible skin expression systems

Conditional, or inducible, gene expression systems have been developed in an attempt to overcome the limitations of constitutive systems. In particular, these systems aspire to provide spatial and temporal control of gene expression. Such control is advantageous for evaluating the role of gene products that are deleterious to development, or, when expressed throughout development, lead to secondary or compensatory effects which complicate their evaluation at later stages.

The ideal conditional system has several important features. First, expression should be completely off until turned on. Second, high levels of the gene product should be rapidly expressed upon full induction. Third, the level of induction should be dependent on the dose of the inducing agent, such that high or low levels of expression can be induced. Fourth, the inducing agent should be deliverable in several formats, in particular, systemically or topically. Fifth, the inducing agent should have no other effects on the animal. And finally, the induced expression should be reversible, so that expression can be turned on and off at will.

To best achieve these goals, most inducible systems for expression of transgenes in skin are bigenic, involving the production of two transgenic mouse lines: an inducer/transactivator/regulator line and a target/inducible line. Several systems which deliver many of the properties of an ideal system have been described, and three systems have become broadly used. The binary systems fall into two categories. The first involves permanent genomic modifications to turn on the transgene, and includes the Cre/loxR or other site-specific recombinase systems from bacteriophage or yeast. The second category includes systems which do not employ permanent genomic modifications, but rather uses transcriptional transactivators which are specific to a target or inducible line driving expression of the transgene. The latter includes tet repressor-based systems from bacteria, and the GAL4/UAS system from yeast. These and other inducible systems for the control of gene expression in the mouse have recently been reviewed (Jaisser, 2000; Lewandoski, 2001; Mills, 2001). The bigenic systems, and in particular their application to skin expression, are described below. A third inducible system is based on the naturally quiescent and inducible nature of the keratin 6 gene in adult epidermis. Although not having all the advantages of the ideal inducible system, the K6 system is monogenic and therefore much easier to generate than bigenic inducible systems.

Keratin 6a (K6a) promoter. The human K6a protein is expressed transiently during normal development, but is then downregulated and turned off in normal adult skin. Expression of K6 is re-initiated upon insult or injury to the epidermis, and can be induced with several chemical agents including phorbol esters and retinoic acid. The promoter region which directs faithful reproduction of the normal pattern of K6 expression in transgenic mice has been described (Ramirez *et al.,* 1998; Takahashi and Coulombe, 1996, 1997). Similar to the endogenous K6 gene, the promoter is inducible by injury, infection, psoriasis, cancer and chemical challenges such as TPA (Mazzalupo and Coulombe, 2001). The K6 promoter drives expression at the surface of embryos at E14.5, and produces significant expression in epithelial structures such as oral mucosa and skin at E15.5 (Mazzalupo and Coulombe, 2001). Embryonic skin expression peaks at E16.5 and is restricted to the periderm, a skin structure which is shed prior to birth, but once the periderm is shed, significant expression is no longer observed. K6 is also expressed at sites of temporary epithelial fusions such as in the eyelids, digits and outer ears (Mazzalupo and Coulombe, 2001). The K6 promoter is not expressed in normal adult skin, but is inducibly expressed (Ramirez *et al.,* 1998; Takahashi and Coulombe, 1996, 1997).

K6 promoter sequences employed for transgenic studies encompass 5.2 kb of 5' regulatory sequences from human K6a gene (Takahashi and Coulombe, 1997), though smaller regions have been used with less consistent results (Takahashi and Coulombe, 1997), or yielded constitutive and non-inducible expression in skin (Mahony *et al.,* 2000). Bovine Keratin 4* (bK4*; the bovine ortholog of human K6) transgenic mice have also been described (Blessing *et al.,* 1993). Reporter genes driven by keratin 6 retained keratinocyte-specific expression in appropriate tissues, including the hair follicle, tongue, footpad, and nail bed. Notably, both human and bovine constructs were constitutively expressed in the outer root sheath and interfollicular epidermis in the absence of any activating stimulus.

Several disadvantages inherent to the K6 system should be kept in mind. Although K6 is not normally expressed and can be induced in adult skin, it is expressed in embryonic development. Thus, expression of the transgene of interest in the embryonic periderm may produce phenotypic effects embryonically which could be deleterious to the development of the mouse line, or may induce developmental effects in the skin which could complicate the interpretation of results in adulthood. Also, it should be noted that upon induction of the K6 promoter with phorbol esters, retinoic acid, or injury, the expression of the transgene occurs on the background of the effects of the inducing agent, which in these examples are not benign. Induction methods create a proliferative or stimulatory state, and thus experiments need to be carefully controlled.

Cre-lox and other recombinase-based systems. Various researchers have developed an irreversible, inducible bigenic system which involves induction of transgene expression by removal of a transcriptional blockade. The most common approach is to remove a polyadenlyated transcription unit by use of the Cre/LoxP or FLPe/FRT recombinase systems (Awatramani *et al.,* 2001; Soriano, 1999). In such systems, two transgenic alleles are crossed: one line drives expression of the recombinase, and the second line responds by expressing the blocked transgene. When the two alleles are combined, action of Cre (or FLPe) excises the transcription unit including its polyadenylation site, allowing expression of a downstream transgene *(Figure 2)*. Such systems can be made specific to the skin by placing the expression of the recombinase under the control of a skin-specific promoter. In theory, the reverse could be done, where the blocked transgene is driven by a skin-specific promoter (or both are skin-specific). However, it is generally considered prudent to limit the expression of recombinases to avoid unwanted modification of the genome via cryptic recombinase sites (Awatramani *et al.,* 2001). The use of inducible or regulated recombinases should limit these untoward effects.

Several methods have been described to make Cre (or FLPe) recombinases regulated by the addition of steroid hormones. The most widely used of these systems is the use of a hybrid molecule comprised of Cre recombinase fused to the hormone-binding portion of the estrogen receptor (termed CreER), or a mutant form of the estrogen receptor which has altered ligand specificity such that it binds tamoxifen rather than estrogen (called CreERT or CreERT2 or CreERT3 depending on which mutant of the estrogen receptor ligand binding domain is employed). The yeast FLP recombinase and its enhanced version FLPe (Buchholz *et al.,* 1998) have also been made similarly regulatable via the fusion to the ligand-binding domains of steroid hormone receptors such as ER (Logie *et al.,* 1998; Nichols *et al.,* 1997).

Although the regulated recombinase systems have the advantage of being quiescent prior to induction, they do not meet all the criteria of a good inducible system. These systems are not ideal for delivering dose-dependent expression of transgenes, and are not reversible because the removal of the transcriptional blockade by the recombinase is a permanent genetic change. Once the recombinase has acted on the genome, the transgene is "on" in the modified cell and all of its progeny. Thus, the transgene expression is an all-or-none event in each cell. It may be possible to achieve graded levels of transgene expression by regulating the number of cells in which recombination occurs. Such graded expression may be useful for the study of secreted molecules.

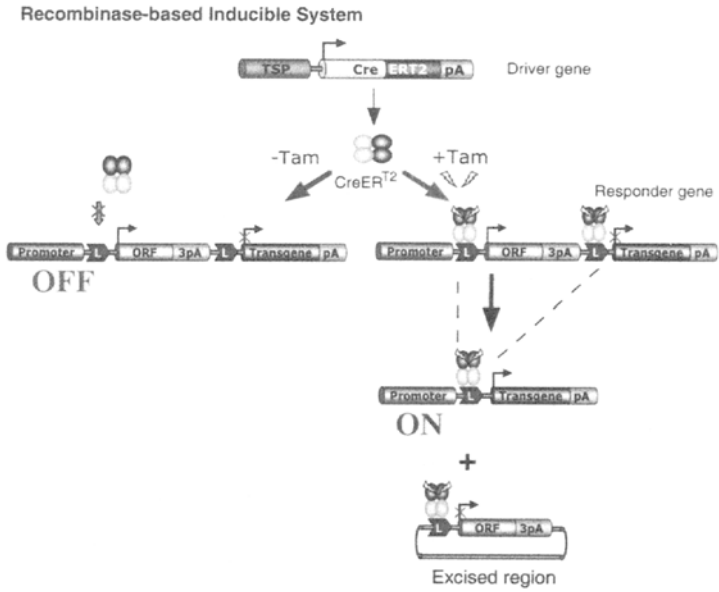

Figure 2. Recombinase system for inducible transgene expression. Shown is the CreER^{T2}-based system, which responds to tamoxifen (Tam) by binding to Lox sites (L) and mediating recombination. In the scheme shown, Cre recombination removes an open reading frame (ORF) and associated polyadenylation sequence (3pA), resulting in transcription of the transgene.

Expression of the recombinase in the basal and stem cell populations of the skin (e.g., under K14) would yield broad and sustained expression of the transgene. As the basal cells give rise to all the other cell types in the suprabasal layers of the skin, these cells should also express the transgene. In comparison, expression of the recombinase in non-stem cells, such as in suprabasal layers (e.g., under involucrin), may lead to temporally restricted expression of the transgene as the cells turn over. Furthermore, if a regulated recombinase such as CreERT2 is expressed in suprabasal cells, transgene expression may diminish over time as the skin cell population turns over. An advantage of such a scenario might be that reinduction of the transgene may be possible upon re-activation of the recombinase. This approach has not yet been tested experimentally.

Several groups have adapted recombinase systems to the skin. In one study (Vasioukhin *et al.,* 1999), transgenic mice were generated which express either Cre recombinase (non-inducible) or a fusion of Cre and the tamoxifen responsive hormone-binding domain of the human estrogen receptor (CreER^{Tam}) under the human K14 promoter. These mice were bred to a line in which a loxP flanked neomycin resistance gene proceeds a promoterless LacZ gene (ROSA26-LacZ (Soriano, 1999)). This reporter construct was integrated into the ROSA26 locus by homologous recombination. Because the ROSA26 gene is expressed in almost all cell types, removal of the loxP flanked neomycin gene results in LacZ expression wherever this modification occurs (Soriano, 1999). In double

transgenic K14Cre/ROSA26-LacZ transgenic embryos, genetically introduced loxP sequences were recombined efficiently and selectively in keratinocytes in skin, tongue, and esophagus. In K14-CreER^{Tam} transgenic mice, loxP sites remained intact until 4 hydroxy-tamoxifen (OHT) was administered. When orally administered, OHT activated keratinocyte-specific CreER^{Tam} throughout the mouse, resulting in recombination of loxP sequences in epidermis, tongue, and esophagus. When topically administered on the skin, OHT activated CreER^{Tam} in the area of skin where tamoxifen was applied. Skin cells harboring a Cre-dependent recombination in their genome persisted for many months after tamoxifen application, indicating that the epidermal stem cell population was targeted (Vasioukhin *et al.,* 1999).

Further refinement of this system is expected. For example, CreER^{T2} was found to be approximately 10-fold better at inducing a transgene in tail skin compared to CreER^{T} (Indra *et al.,* 1999). CreER^{72} requires lower amounts of OHT, thus making it more suitable for use in embryos. Other investigators have generated lines of mice driving a truncated version of the progesterone receptor (PR1) fused to Cre. Progesterone receptor PR1 fusions do not bind to progesterone, but bind and are activated by anti-progestins such as RU486. CrePR1 was expressed in skin (using K5 and K14 promoters) (Berton *et al.,* 2000; Cao *et al.,* 2001). These lines have been used to generate an inducible mutant Keratin 14 gene product, which results in a skin-blistering phenotype (Cao *et al.,* 2001), and have been mated to another line to achieve inducible activation of a somatic Keratin 10 mutation at the K10 locus (Arin *et al.,* 2001). CrePR1 may not be suitable for induction of transgenes prior to birth because the antiprogestins used to induce it can lead to abortion of the embryos, however it has recently been reported that co-administration of progesterone with RU486 can lead to the survival of embryos and the induction of a reporter transgene in skin when injections are initiated at E14.5 (Cao *et al.,* 2002; Zhou *et al.,* 2002). Unlike some other inducing agents such as OHT, RU486 has very low toxicity in adults, and thus can be used at high levels over long periods of time(Wang et al., 1999).

Because the Cre/LoxP and other recombinase systems described above involve two separate transgenic lines, they can be very versatile and modular. For example, the responder transgene line can be bred to multiple Cre driver lines to vary the patterns of transgene expression. Depending on the promoter driving the expression of recombinase, one could get transgene expression only in suprabasal cells, or in basal cells and their progeny. A further advantage is that induction of expression in skin can be achieved in several ways, including by systemic or topical skin administration of the steroid hormone or analog.

To reduce the amount of breeding, one improvement would be the generation of a monogenic system in which the recombinase and the transgene are expressed at the same locus. Such a modification could still allow the breeding of such alleles to other recombinase lines, and thus may still retain some of the versatility afforded by the bigenic systems.

When using the recombinase systems, it is important that the target transgenic mice be well characterized. In particular, it is critical that only a single copy of the LoxP-flanked transgene be present in the genome. In cases of multiple LoxP sites, activation of Cre can

result in a variety of rearrangements. Because pronuclear injection methods frequently result in multiple or random integrations, the action of Cre would be unpredictable and variable.

Gene switch system. The gene switch system represents a modification of a bigenic expression system used commonly in Drosophila (Roman *et al.,* 2001), but which also functions well in mice (Wang *et al.,* 1999). Like other inducible systems, this basic system involves a driver (transactivator) line and an inducible responder line *(Figure 3)*. The driver line directs expression of the transactivating protein GAL4 from yeast in a tissue-specific or inducible fashion (or example, under the control of a heat shock promoter in drosophila (D'Avino and Thummel, 1999)). The inducible line employs a basal promoter with one or more GAL4binding sites (UAS sites). The inducible line is off until the UAS sites are bound by the GAL4 transactivator. Although this system has been used in only a small number of transgenic mouse lines to date, it appears to work well. It has not gained popularity due to the difficulty in regulating activity of the GAL4 transactivator in vivo.

Wang and collegues made a clever innovation to this system (gene "switch" system) by fusing the GAL4 transactivator to a truncated hormone-binding region of the progesterone receptor *(Figure 3)* (Wang *et al.,* 1999). Further, to increase the transactivating potential, the herpes virus VP16 transactivating domain was fused to the C-terminal end of the truncated progesterone receptor to make a tripartite chimeric protein, termed GLVPc. By use of GLVPc, the production of a functional transactivator is dependent on binding of antiprogestins, such as RU486, to the hybrid molecule. Activation of the transgene does not occur until GLVPc binds a small molecule, which can be provided systemically or readily applied to the skin. A transgene can be induced focally in the epidermis at different levels of expression levels or at different developmental stages (Wang *et al.,* 1999). Because there are no UAS sequences in the mammalian genome, there is very low non-specific induction of other genes. This system has been used to establish a viable transgenic model for studying functions of TGFβ 1 in the skin

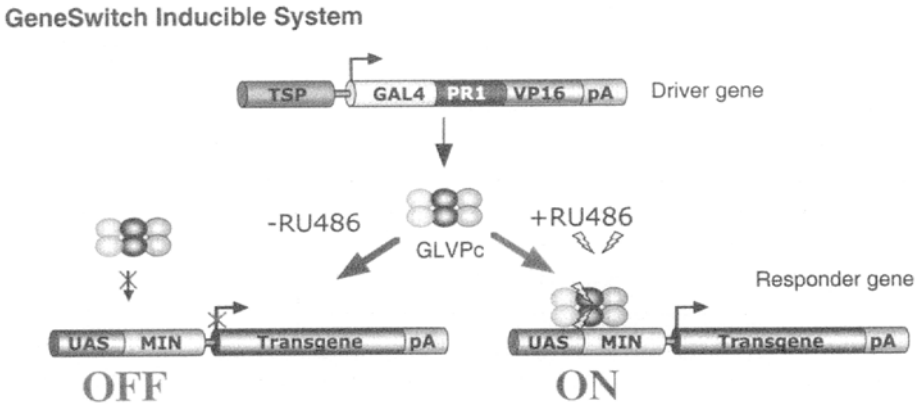

Figure 3. GeneSwitch system for inducible transgene expression. The GLVPc acts as the driver gene. Upon binding RU486, the GLVPc gene product binds to UAS regions and induces transcription of the transgene.

(Wang *et al.,* 1999). In the system described (Wang *et al.,* 1999), the driver line employs the mouse loricrin promoter, which drives expression of the transactivator GLVPc. In the inducible responder line, a thymidine kinase promoter with four upstream GAL4-binding sites regulates the transgene of interest. These two transgenic lines were mated to generate bigenic mice, and expression of the transgene was controlled by topical application of an antiprogestin (Wang *et al.,* 1999). Recently an enhanced version of the GeneSwitch for driving transgenes in skin has been reported (Cao *et al.,* 2002). This study uses a new version of the activator in which VP16 is replaced with that of the NFkB p65 subunit. This gave lower baseline expression levels and higher inducibility (Cao *et al.,* 2002).

Tetracycline-based systems. A very widely applied approach for conditional transgene expression uses the tetracycline-regulated systems initially described by Gossen and Bujard (Baron *et al.,* 1997; Gossen and Bujard, 1992). The two components of this system include a transactivator driver transgene composed of a hybrid molecule between the herpes virus transactivator VP16, and the Tet repressor protein from bacteria. This hybrid protein binds to tetracycline (or doxycycline—Dox) and then to DNA elements alternately termed tetracycline response elements (TRE), or tet operator sequences (TetO). When TRE/tetO sequences are placed upstream of a minimal promoter they can lead to tetracycline-dependent transcriptional activation of transgenes in the responder line *(Figure 4)*.

There are two versions of this system which have become popular, termed the tet ON and the tet OFF systems *(Figure 4)*. In the tet OFF system, tetracycline (or doxycycline—Dox) bound to the transactivator protein (tet transactivator, "tTA") prevents it from binding DNA and transactivating the target gene. Gene induction occurs in this sytem upon withdrawal of tetracycline. In the tet ON system, a mutant version of the tet transactivator, the "reverse tet transactivator" (rtTA) is utilized which binds TRE/tetO sequences only when bound to tetracycline.

The main practical differences in these two systems is in their manner of induction. When using the tet OFF system, animals are kept on Dox to maintain the transgene in the off state. Transgene induction is achieved by withdrawal of Dox, and thus the time to induction is gradual and dependent on the time of clearance of Dox from the target tissue. Turning the transgene back off again can be done more rapidly by the reintroduction of Dox. The tet ON system is simpler—animals are given Dox to induce the transgene. Transgene induction is thus much more rapid than in the tet OFF system, but downregulation thereafter is slow and dependent on the clearance time of Dox in the target tissue.

In both tet ON and tet OFF systems, two lines of mice must be generated and carefully characterized prior to mating. A critical factor is to establish a mouse line in which the responder transgene is truly OFF in the baseline state. Both systems have additional complications. In the tet OFF system, mice must be maintained on Dox throughout their lives to keep the transgene off, and induction can be slow when Dox is withdrawn. In principle the tet ON system is the most desirable of the two, however, in practice it has been challenging to get to work appropriately. One possible reason for this is residual

affinity for TRE/tetO activity of rtTA even in the absence of Dox. In addition rtTA is relatively inefficiently induced by Dox.

Recently, rtTA proteins with improved properties were screened for after mutagenesis in *Saccharomyces cerevisiae* (Urlinger *et al.,* 2000). One new version, termed rtTA2^{s}-M2, functioned at a 10-fold lower concentration of Dox, was more stable in eukaryotic cells, and caused no background expression in the absence of Dox. This molecule was subsequently codon optimized for expression in mammalian cells. rtTA2^{s}-M2 was shown to have excellent properties in mammalian cell culture (Urlinger *et al.,* 2000), but its use in mouse models has not yet been reported. It has also been reported that high levels of tTA or rtTA can be toxic to mammalian cells due to general transcriptional squelching from the hsv VP16.

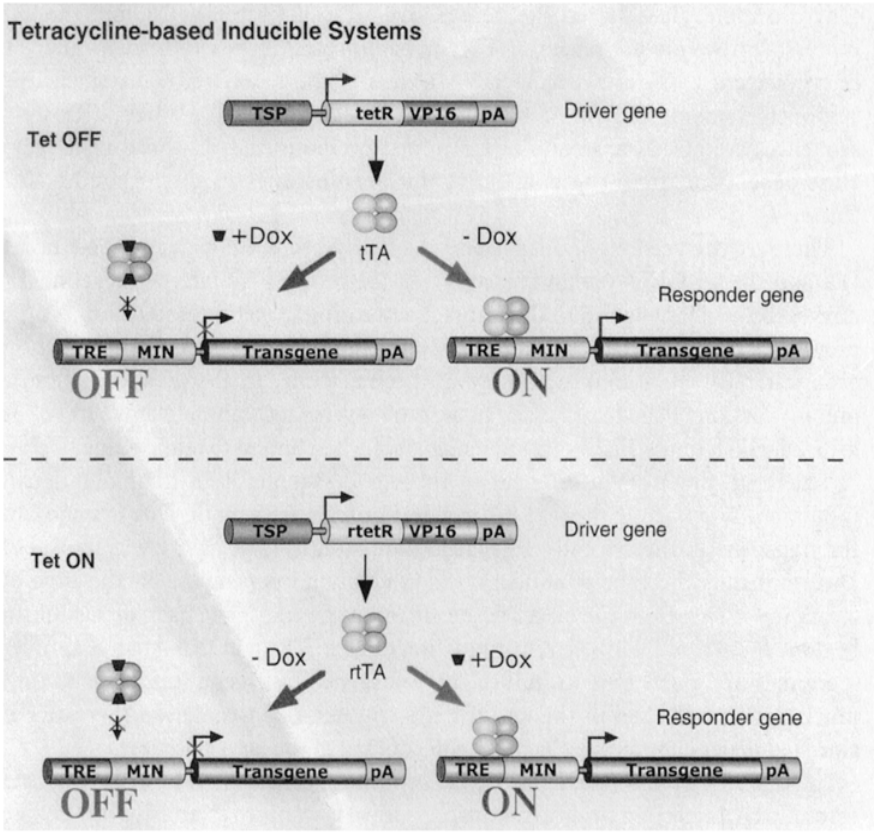

Figure 4. Tetracycline system for inducible transgene expression. In the tet OFF scheme, binding of doxicycline (Dox) by tTA protein prevents binding to the promoter and keeps the transgene off. Upon binding Dox, tTA binds to the Tre promoter and induces transcription of the transgene. In the tet ON scheme, binding of Dox by rtTA protein allows binding to the promoter and induces transcription of the transgene.

Several groups have used tetR systems for expression of transgenes in skin. In one study, the bovine K5 promoter was used to drive either tTA or rtTA (Diamond *et al.,* 2000), and a constitutively active mutant of TGFβ1 was linked to the TRE/tetO (Liu *et al.,* 2001). Prior to breeding, candidate founders from the TGFpl line were screened for low basal expression and degree of induction by first isolating primary keratinocytes from newborn animals and transfecting them with the K5-rTA plasmid. Cells were assayed for the production of transgene before and after treatment with Dox in culture (Liu *et al.,* 2001). This approach may be valuable to assure that the appropriate target lines are found early in the process. In bigenic mice containing both the K5-rtTA and TGF(31 transgenes, induction of transgene was detected in skin within 24 hours of treatment, and apparently reached maximal levels by 48 hours. In the tTA line, transgene expression became highly induced 2 to 3 weeks after removal of Dox, and dramatic phenotypic changes could be observed at 3 to 4 weeks (Liu *et al.,* 2001). This system functioned as expected in terms of achieving dose-related induction of transgene expression *in vivo,* and phenotypic effects were reversible in tTA mice within 7 days after readministration of Dox.

A second study used a similar strategy to induce expression of the ErbB2 oncogene in skin (Xie *et al.,* 1999). In this case, the transactivator line utilized the human K14 promoter driving expression of rtTA (Xie *et al.,* 1999). In bigenic animals, ErbB2 transgene expression could be detected within 4 hours and was maximal within 24 hours after application of Dox, whereas phenotypic effects could be detected as early as 48 hours (Xie *et al.,* 1999). Phenotypic changes could be rapidly reverted to normal by withdrawal of Dox, even after several days of administration. These studies reveal the power of the tetR systems for induction of transgene expression in an inducible and dose-dependent manner in basal keratinocytes. Neither study used local/topical delivery of Dox to skin, however systemic administration seemed to work well for skin expression.

5.
Vascular phenotype of transgenic mice

5.1
VEGF-A

Several groups have generated mice which overexpress VEGF-A in the skin, under K14 (Detmar *et al.,* 1998; Thurston *et al.,* 1999) or K5 promoters (Larcher *et al.,* 1998). The skin phenotypes of these different lines of mice are similar, although differences in the severity of the phenotype are apparent. The differences in severity likely reflect differences in transgene copy number and expression levels in the different founder lines. In general, these mice all show increased vascularity in the dermis, as well as evidence of inflammation and edema, including thickening of the dermis. These features dramatically support the previously observed dual functionality of VEGF, in particular, its actions as an endothelial cell growth factor and a vascular permeability factor (Dvorak *et al.,* 1995).

The increased vascularity in the skin of the VEGF transgenic mice is a result of increased numbers of small capillary-sized vessels subjacent to the epidermis and

surrounding the hair follicles. *In situ* hybridization for VEGF reveals that the basal cells of the epidermis, and the epithelium surrounding the hair follicles, are sites of strongest VEGF expression, thus the sites of vascularity match the location of VEGF expression. VEGF was not increased in the systemic circulation, suggesting that the transgenically produced VEGF remained in proximity to the site of production, or that levels of expression were not sufficient to increase systemic VEGF. In one study (Detmar *et al.,* 1998), abundant mast cells were found in the dermis, and the skin vessels supported increased leukocyte rolling which was blocked by antibodies to E-and P-selectins. Increased leakage of plasma proteins in the skin was also found under baseline conditions (Detmar *et al.,* 1998: Thurston *et al.,* 1999).

Interestingly, the epidermis of the K14-VEGF mice was thickened, and, in one strain of mice, lesions appeared in the skin of older mice (Thurston *et al.,* 1999). These lesions were associated with hyperkeratosis and parakeratosis (A.Xia and J. Rudge, unpublished). The abnormalities in the epidermis reveal interplay between the vascular and epithelial compartments of the skin. In particular, the action of VEGF on the blood vessels appears to be able to feed back to the epidermis. The VEGF transgenic mice highlight the role of VEGF as a potent angiogenic factor, but also emphasize that the resultant vessels can be leaky and inflamed.

5.2
Angiopoietin-1

Mice over-expressing Angiopoietin-1 (Ang1) under the K14 promoter have been produced (Suri *et al.,* 1998). The skin of K14-Ang1 mice appears very reddened, and the dermal vessels were increased in diameter compared to normal mice, but only moderately increased in number. The enlarged vessels had an increased number of endothelial cells, indicating that Ang1 induced increased endothelial cell proliferation or survival. The enlarged vessels were in the position of capillaries subjacent to the epidermis and surrounding the hair follicles, but had the size and certain phenotypic properties of venules (Thurston *et al.,* 1999). For example, the endothelial cells had immunoreactivity for P-selectin, which in the skin is normally expressed in venular endothelial cells but not by those in capillaries or arterioles. As with the K14-VEGF mice, circulating levels of Ang1 were not found, and the effects on the vasculature were localized to the sites of Ang1 overexpression.

The K14-Ang1 mice were compared side-by-side with mice overexpressing VEGF (Thurston *et al.,* 1999). Although both strains of mice have increased vascularity in the skin, the appearance and function of the resultant vessels are very different. Unlike the K14-VEGF mice, the dermis and epidermis of K14-Ang1 is normal in thickness and does not contain infiltrating mast cells or other leukocytes. The vessels in K14-Ang1 mice are not leaky under baseline conditions, and remarkably, seem to be resistant to plasma leakage induced by inflammatory mediators such as histamine, serotonin, and mustard oil.

5.3
Double transgenic VEGF/Angl mice

K14-Ang1 mice were bred to K14-VEGF mice (Thurston *et al.,* 1999). The skin of the resultant double transgenic K14-Ang1/VEGF mice is dramatically reddened, and the vascularity of the skin is higher than either K14-VEGF or Ang1 mice. The morphology of the vessels appears to be a combination of the Ang1 and VEGF effects—in particular, numerous small vessels and enlarged vessels are both present (Thurston *et al.,* 1999). In terms of vessel function, the actions of Ang1 seem to prevail: the dermis of K14-Ang1/VEGF mice is normal in thickness and does not contain increased numbers of leukocytes. In addition, the vessels are not leaky under baseline conditions. Thus, Ang1 seems to inhibit some of the inflammatory actions of VEGF, however Ang1 and VEGF appear to act on distinct signaling pathways for vessel growth. These double transgenic mice emphasize the complementary actions of VEGF and Ang1 on blood vessels.

5.4
Hypoxia inducible factor

Hypoxia inducible factor 1 alpha (Hif-1α) is a key element of the cellular response to decreased oxygen concentration (Semenza, 2001). At normal oxygen (above 5%), Hif1α is unstable and is constitutively degraded by the ubiquitin pathway. Below 5% oxygen, Hif1α is stabilized, and can bind to other factors to form a transcription factor. The domain of Hif-1α that is responsible for oxygen-mediated degradation is known as the oxygen degradation domain (ODD). Several genes have been identified which are transcriptionally upregulated by hypoxia and Hif-1α, by virtue of a defined promoter element known as HRE (hypoxia response element). The upregulated genes include glucose transporters and other proteins involved in metabolism, but, for the purposes of this chapter, a key upregulated gene is VEGF. A constitutively active form of Hif-1α, which lacks the ODD domain (Hif-1αΔODD), can increase VEGF expression even in normal oxygen.

Transgenic mice were generated which over-express either Hif-1α or Hif-1αΔODD under the K14 promoter (Elson al., 2000). The skin of K14-Hif-1α mice appears normal, with normal levels of VEGF expression in the epidermis, and normal vasculature. In contrast, the skin of K14-Hif-1αΔODD mice appears reddened, and VEGF expression is increased several-fold in the epidermis. The vascularity of the skin of K14-Hif-1αΔODD mice is dramatically increased, with a plexus of capillary-sized vessels subjacent to the epidermis and baskets of vessels around the hair follicles (Elson *et al.,* 2000). The appearance of the vessels in the K14-Hif-1αΔODD mice is somewhat similar to that of K14-VEGF mice, although the vessels appeared to be less tortuous in the K14-Hif-1αΔODD mice. The similarity of the vessels is consistent with the increased expression of VEGF However, the function of the vessels in the K14-Hif-1αΔODD and K14-VEGF mice is very different. Unlike the K14-VEGF mice, the skin in the K14-Hif-1αΔODD mice was normal in thickness, and the vessels did not leak under baseline conditions.

To determine whether the normal function of the vessels in the K14-Hif-1αΔODD mice was due to concomitant upregulation of Ang1, the skin was tested for expression of Ang1. Ang1 mRNA was not increased. However, unlike K14VEGF mice in which only one isoform of VEGF (164 kD) is overexpressed, K14Hif-1αΔODD mice express various isoforms of VEGF, including 121, 164, 188, and 209 kDa (Elson *et al.,* 2000). Thus, the normal blood vessels in K14-Hif-1αΔODD mice may be due to the concomitant expression of various isoforms of VEGF, or to the co-expression of other, unidentified factors. These Hif-1α transgenic mice emphasize that the program for endogenous angiogenesis is more complex than merely upregulating one VEGF isoform.

5.5
Lymphatic growth factors—VEGF-C, VEGF-C165S, soluble VEGF-R3

The skin contains abundant lymphatic vessels, and the skin transgenic system has been used to elegantly demonstrate the role of VEGF-C and VEGF-R3 in formation of lymphatic vasculature. K14-VEGF-C mice were generated using standard approaches (Jeltsch *et al.,* 1997). These mice had thickened skin and enlarged lymphatic vessels, without increases in the size or number of blood vessels. The enlarged lymphatic vessels appeared to have more proliferating endothelial cells than lymphatic vessels in normal mice. These mice established that VEGF-C could induce lymphatic vessel growth in vivo.

To address whether VEGF-C was acting on VEGF-R2 or R3, skin transgenic mice were produced which expressed VEGF-D or a form of VEGF-C (VEGFC156S) which binds only to VEGF-R3 (Veikkola *et al.,* 2001). VEGF-D was found to also increase the lymphatic vasculature in the skin. In addition, VEGF-R3 was identified as the key receptor, because K14-VEGF-C156S mice also had increase skin lymphatics. The blood vessels in these transgenic mice were not affected, suggesting that VEGF-R3 and its ligands act specifically on the lymphatic vasculature, at least when expressed from the K14 promoter.

A soluble form of VEGF-R3 receptor, VEGF-R3-Ig, has been shown to inhibit lymphangiogenesis induced by VEGF-C (Veikkola *et al.,* 2001). To further explore the effects of this soluble receptor, K14-VEGF-R3-Ig mice were generated (Makinen *et al.,* 2001). These mice have a thickened dermis and subcutaneous layer. The lymphatic vessels were largely absent from the skin of K14-VEGF-R3Ig mice, but the blood vessels appeared normal. The soluble receptor was detected in the serum of the transgenic mice, and appears to have a transient inhibition of formation of the lymphatic vessels in internal organs. The transgenic mice had very few lymphatic vessels in heart, esophagus, lungs, and other organs at age 2 weeks. In adult mice, the lymphatic vessels had largely recovered in the internal organs of transgenic mice, despite continued presence of VEGF-R3-Ig in the serum. It is not known how the lymphatic vessels seem to escape their need for VEGF-C.

5.6
Hepatocyte growth factor/scatter factor

Hepatocye growth factor/scatter factor (HGF), the ligand for c-Met receptor tyrosine kinase, has growth and motility effects on various cell types, and may play a role in some human cancers, including cutaneous melanoma (Noonan *et al.,* 2000). HGF also has angiogenic actions. Although the c-Met receptor is expressed by various cell types, HGF may promote angiogenesis by acting directly on endothelial cells, or it may interact with other vascular growth factors. HGF was transgenically overexpressed in numerous cell types under the metallothionein promoter (Takayama *et al.,* 1996; Toyoda *et al.,* 2001). The resultant mouse skin has been analyzed in detail, and appears to have a different distribution of melanocytes than normal mouse skin (Noonan *et al.,* 2000). Furthermore, the skin has an abnormal wound healing response. Wound sites accumulate increased granulation tissue and contain increased numbers of blood vessels. The increased vascularity is associated with increased expression of VEGF in the transgenic skin, especially during wound healing (Toyoda *et al.,* 2001).

5.7
Thrombospondin1

Thrombospondins (TSPs) are matrix glycoproteins that regulate cellular attachment, migration, proliferation, and differentiation. TSP-1 inhibits endothelial cell proliferation and migration in tissue culture, and is a putative inhibitor of angiogenesis in vivo. To further test the functions of TSP-1 in vivo, transgenic K14-TSP-1 mice were generated (Streit *et al.,* 2000). The skin of these mice appeared normal and healthy. In addition, the numbers and morphology of the dermal blood vessels were normal, and the vessels had a normal leakage response to VEGF. However, the time to healing of full thickness skin wounds was delayed in K14-TSP-1 mice. The delayed healing was associated with a delay in formation of granulation tissue and with reduced vascularity of the granulation tissue. The reduced vascularity seemed to be due to a reduced size (diameter) of the blood vessels, rather than a reduction in the number of vessels. The authors concluded that overexpression of TSP-1 resulted in a defect in wound repair angiogenesis, but not normal developmental angiogenesis.

5.8
TGF-b1

TGF-β1 is believed to be an important, albeit complex, regulator of vascular growth (Antonelli-Orlidge *et al.,* 1989; Darland and D'Amore, 2001). Transgenic mice were produced in which an active form of TGF-β1 was expressed under the keratin-1 promoter (Sellheyer *et al.,* 1993), however these mice died neonatally, making study of the blood vessels difficult. More recently, a "gene-switch" transgenic system was used to inducibly overexpress TGF-β1 in the epidermis of adult mice (Wang *et al.,* 1999). Induction of the transgene by topical application of a compound, which binds progesterone receptor, ZK,

resulted in decreased proliferation of epidermal cells and aberrant expression of keratinocyte markers. In contrast, TGF-β1 induction for 1 week resulted in markedly increased vascularity, characterized by enlarged blood vessels near hair follicles and subjacent to the epidermis. It is not known whether this increased vascularity was a direct action of TGF-β1 on the blood vessels, or via induction of other factors such as VEGF (Wang *et al.,* 1999). These mice represent one of the first applications of inducible skin transgenics to study blood vessels, and will be an important tool to understand the vascular actions of a complex signaling system.

Transgenic mice expressing dominant negative TGF-β receptor II under the mouse loricrin promoter (mL-ΔTGF-βRII) were produced to examine the role of this receptor in cancer (Wang *et al.,* 1997). The epidermis of these mice is thickened at birth, suggesting a decrease in growth suppression, but otherwise the skin is fairly normal. However, the skin of these mice is very sensitive to chemical carcinogenesis. Application of a mutagen plus phorbol ester to the skin resulted in increased formation of carcinomas associated with angiogenesis and metastases (Go *et al.,* 1999). The increased angiogenesis was associated with increased expression of VEGF and decreased expression of thrombospondin-1.

5.9
Inflammatory factors

Many inflammatory and immunologic cytokines have been used to make skinspecific transgenic mice (Yang *et al.,* 2000). For example, skin transgenic mice overexpressing IFNγ, TNFα, IL-4, and IL-6 have been found to exhibit skinspecific inflammatory reactions (Carroll *et al.,* 1997; Chan *et al.,* 2001; Cheng *et al.,* 1992; Mehling *et al.,* 2001; Turksen *et al.,* 1992). Although these inflammatory reactions are likely to be associated with vascular remodeling and angiogenesis, most reports to date give only passing reference to the vasculature (for example, IFNγ (Carroll *et al.,* 1997)). One exception is a study of skin-transgenic mice overexpressing IP-10 (Luster *et al.,* 1998), a factor that may be involved in T-cell recruitment. These transgenic mice had abnormal wound healing in skin, including reduced angiogenesis in the resultant granulation tissue.

6.
Transgenic mice with skin carcinogenesis and angiogenesis

Many transgenic lines of mice have been created in which a keratinocyte promoter is driving a protein that acts on the epithelium, either in an autocrine or paracrine fashion. Such mice include K14-TGFα (Vassar and Fuchs, 1991), K5-E1a (Missero *et al.,* 1993), and K5-PKC-α (Wang *et al.,* 1994). Such mice have shed light on growth regulation of the epithelium, but have less relevance for angiogenesis. However, transgenes which act on the epithelium to produce a hyperplastic or neoplastic epidermis, and thereby induce stages of carcinogenesis, can result in indirect effects on the dermal blood vessels. Indeed, the angiogenic phenotype has been well described in two such lines, K14-HPV16 and involucrinc-myc, and these will be summarized here.

6.1
Papilloma virus oncogenes

The early genes from human papillomavirus 16, coupled to the keratin-14 promoter, were used to generate skin-specific transgenic mice (K14-HPV16) (Arbeit *et al,* 1994). In the FVB/n background, these mice display multistage epidermal carcinogenesis, characterized by a progression from hyperplasia, dysplasia, and finally invasive squamous carcinomas (Arbeit *et al.,* 1996; Coussens *et al.,* 1996). The stages of carcinogenesis are associated with changes in the dermal blood vessels. Notably, there is a dramatic increase in the number of blood vessels subjacent to the epidermis during the late dysplastic stage (Smith-McCune *et al,* 1997). The induction of new blood vessels is associated with an increase in the amount of VEGF mRNA in the dysplastic epidermis, as detected by in situ hybridization (Smith-McCune *et al.,* 1997).

The link between the hyperproliferation of the epithelium in the K14-HPV16 mice and the onset of angiogenesis appears to be due to recruitment of inflam matory cells and induction of protease activity. Specifically, mast cells and metalloproteinase MMP-9 (gelatinase B) are associated with the progression of carcinogenesis in the K14-HPV16 model. The number of mast cells is increased in the skin of K14-HPV16 mice, and they were found in close proximity to the angiogenic vessels in dysplastic skin lesions. The activity of the mast cell proteinases mMCP-4 and-6 was induced in the lesions, as was the downstream proteinase MMP-9. In an heroic effort, K14-HPV16 mice were crossed with mice deficient in mast cells (KIT^{w}/KIT^{Wv}) to generate a very small number of K14-HPV16: KIT^{w}/KIT^{Wv} mice. These mice had mildly increased proliferation of the keratinocytes, but failed to progress to the angiogenic dysplastic stage (Coussens *et al.,* 1999). Similarly, mice deficient in MMP-9 show reduced keratinocyte proliferation and failed to progress to the angiogenic dysplastic stage (Coussens *et al,* 2000). Thus, the proliferation of epithelial cells can induce angiogenesis in the underlying dermis, but the action of the inflammatory system appears to be required to link the two tissue compartments.

6.2
c-myc oncogene

The protooncogene *c-myc* has been coupled to skin-specific promoters to generate several different lines of transgenic mice (Arnold and Watt, 1999; Waikel *et al.,* 1999,2001). In one approach, a fusion protein was created to provide switchable expression of c-Myc activity (Arnold and Watt, 1999; Pelengaris *et al.,* 1999). The hormone-binding domain of a modified estrogen receptor (ER^{TM}) was fused to c-Myc to produce a protein that is normally degraded, but, in the presence of tamoxifen, is stabilized and becomes active. Transgenic mice were made in which this construct is driven by the suprabasal involucrin promoter (Pelengaris *et al.,* 1999).

Topical application of tamoxifen to the skin of involucrin-c-myc-ER^{TM} mice induced c-Myc activity and epidermal cell proliferation (Pelengaris *et al.,* 1999). Continued application of tamoxifen resulted in hyperplasia and dysplasia of the epidermis, and, by 1

week, angiogenesis in the dermis. The new blood vessels were prominent in the dermal papillae. Activation of c-Myc appeared to induce increased expression of VEGF by the keratinocytes, which may account for the angiogenic activity. It is not known whether c-Myc activation also results in activation of mast cells and the inflammatory system. Thus, activation of c-myc appears to produce another model of skin carcinogenesis and associated angiogenesis.

7. Discussion

The use of skin transgenic mice has produced several resounding successes in terms of increasing our understanding of the actions of angiogenic factors. Overexpression of VEGF-A in the skin confirmed and extended our understanding of the dual functionality of this factor, which can serve as both an angiogenic and a leakage factor. Ongoing studies with VEGF transgenic mice are giving new insights into the potential role of VEGF in chronic inflammatory diseases. Similarly, overexpression of Ang1 confirmed that it is indeed a vascular-specific factor, and in addition, revealed an unexpected anti-leakage action. The Ang1 overexpressing mice, and the double transgenic VEGF-Ang1 mice, served to contrast the actions of VEGF and Ang1. In addition, upstream induction of angiogenesis by overexpression of active Hif1α, emphasized that artificial overexpression of one isoform of VEGF-A does not mirror the endogenous program of angiogenesis. These Hif1α transgenic mice may teach us about realistic approaches to pro-angiogenic therapy. In another set of studies, skin-transgenic mice were elegantly used to confirm the role of VEGF-C and VEGF-R3 in lymphatic vessel growth.

Several other secreted factors—both pro-and anti-angiogenic—await confirmation in skin transgenic systems. Such factors include: angiostatin (O'Reilly *et al.,* 1994), endostatin (O'Reilly *et al.,* 1997), angiopoietin-related factors (Peek *et al.,* 2002), various MMPs and their inhibitors, and EG-VEGF (LeCouter *et al.,* 2001). As more factors are analyzed in the skin transgenic system, our understanding of what constitutes pro-and anti-angiogenic factors is likely to evolve. For example, we may be able to distinguish, *in vivo,* whether factors induce vascular sprouting or vascular enlargement without sprouting. Obviously, interpretation of the results is simplified when the actions of the factor are specific to vascular cells.

A recurring theme in skin transgenic models is the need to subject the skin and the vasculature to some sort of challenge assay in order to see a phenotype. Such challenge assays include wound healing and induced carcinogenesis. As our understanding of the structure and biology of the skin vessels increases, it is likely that such challenge assays will be increasingly utilized and refined.

As the molecular tools evolve, we will undoubtedly move beyond the relatively simple transgenic systems in which one cDNA is produced at constant high levels throughout the lifetime of the mouse skin. Transgenic systems will become more sophisticated by employing inducible systems, generating multiple growth factors, exploring more upstream intracellular regulatory factors, and refining the models of skin and vascular diseases.

As genomic sequence becomes fully available, and the hunt intensifies to identify the functions of newly identified genes, the skin transgenic system may become a screening system in which mouse lines are rapidly produced and examined in standardized assays for phenotype. While such an approach seemed unnecessary and unlikely even a few years ago, rapid developments in molecular genetics, and the appreciation of just how difficult it is to really understand the actions of novel factors, make such a scenario possible.

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9
Genetics of vascular malformations

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1. Introduction

Appropriate formation of the vascular system is essential for life. Numerous knockout mouse models have been used to define molecules that contribute to the intricate pathways of angiogenesis and vasculogenesis. Resultant early embryonic lethal phenotypes due to impaired vascular or cardiac morphogenesis identify molecules essential for the earliest stages of vascular development. The human vascular defects which create specific, localized defects in the blood vessels provide a complementary approach since the genetic defects that generate these progressive lesions which develop with time have the potential to define finer regulators of integrated responses. Investigations into the genes involved in such inherited malformation syndromes are allowing parallels to be drawn between conditions resulting in apparently disparate vascular endpoints. Furthermore, understanding these pathophysiological mechanisms also permits insights into the processes that maintain, remodel and adapt the vasculature to the stresses encountered in adult life.

The majority of vascular malformations occur in only a small proportion of vessels that express the defective gene. The mechanisms by which a single gene mutation present in all endothelial cells leads to very distinct lesions, which evolve with time are protean, and provide fundamental insights into stresses experienced by endothelial cells during their lifetime. Turbulent blood flow due to perturbation of the hypothetical laminar flow in an idealized cylindrical vessel, compounded by decreases in supportive extracellular matrix were highlighted by the first arterial diseases to reveal their genetic basis. These included Marfan syndrome *(fibrillin-1)* (Dietz *et al.,* 1991) and Ehlers Danlos syndrome type IV (*COL3A1* encoding a type III collagen chain) (Pope, 1993). Malformations in regions of the circulation that are subject to lower transmural pressures and shear stress should highlight potential triggers of non-mechanical natures, including inflammation, repair, and additional somatic genetic changes. Understanding the triggering of malformations under

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these circumstances is likely to further illuminate our appreciation of vascular development and pathophysiology.

1.1
Insights from vasculogenesis and angiogenesis

As described in detail elsewhere, differentiation of intraembryonic progenitor endothelial cells from lateral plate and paraxial mesoderm is a prelude to the establishment of vascular structure in the embryo in vasculogenesis. Yolk sac hemangioblasts defined by the expression of VEGF-R2/Flk1 in addition to CD34 and CD31 migrate into extraembryonic tissues and aggregate into cords of cells prior to the separation of hematopoietic and endothelial cell lineages as lumens develop. Intraembryonic $TAL1^+$/$Flk1^+$ angioblast precursors (Drake and Fleming, 2000) arise as individual cells which proliferate, extend sprouts and interconnect to form the primary vascular plexus. Subsequent fusion and remodeling leads to the formation of the early intraembryonic vessels, which express additional endothelial cell-specific markers including the receptor tyrosine kinase Tie2. Vasculogenesis has been shown to continue in adults, with progenitor cells being mobilized from bone marrow tissue (Asahara *et al.,* 1999). The development of new vessels through sprouting from pre-existing vascular structures in angiogenesis highlights the need for coordinate regulation of endothelial cell activation, detachment, proliferation and maintenance of viability in evolving structures devoid of usual stabilizing signals. Vascular integrity also requires recruitment and differentiation of vascular smooth muscle cells and pericytes, the establishment of a supportive and strengthening extracellular matrix, and coordinate regulation of the molecules which provide signaling networks between all of these components in angiogenic remodeling.

Participants in any of these vasculogenesis, angiogenesis and angiogenic remodeling pathways could provide possible candidate genes to be mutated in inherited vascular malformation syndromes. However, it should be recognized that for vascular malformations to develop, any genetic defect must have a sufficiently minor effect on overall vascular development to permit the development of a viable organism. Even where malformations are congenital, they will often occur within an initially appropriately formed vascular bed. Causative defective genes are therefore likely to highlight molecules in which deficiencies or inappropriate activities have more deleterious effects on angiogenesis and angiogenic remodeling processes of existing vascular structures than vasculogenesis. Such processes may be perturbed when occurring as part of normal development, repair or during adaptations to environmental changes such as by compensatory mechanisms to supply blood to hypoxic tissues including wounds and tumors.

In this chapter we focus on malformations that implicate specific signaling pathways involved in different stages of vasculogenesis and angiogenesis, particularly where these highlight disparate mechanisms that act as triggers for the fully mature malformations. Vascular malformations are divided clinically into groups according to which section of the vasculature is predominantly affected such as arterial, capillary, venous, or

arteriovenous. Although primarily an anatomical classification, these terms serve as reminders of the likely shared stresses and triggers for the resultant malformations.

2. Considerations from aberrant signaling in early vasculogenesis

The endothelial cell mitogen vascular endothelial cell growth factor (VEGF-A) and its cognate high-affinity endothelial cell-specific receptor tyrosine kinases flt1/VEGF-R1 and flk-1/VEGF-R2 might be suspected to be ideal candidates for vascular malformation syndromes. However, they are essential for such early stages of vasculogenesis that even heterozygous loss of one VEGF allele is incompatible with life (Carmeliet *et al.,* 1996; Ferrara *et al.,* 1996). Perturbment of initial differentiation of angioblasts by aberrant VEGF-R2 signaling (Shalaby *et al.,* 1995), or deranged assembly of the primitive capillary plexus due to defects in the VEGF-R1 axis (Fong *et al.,* 1995) might be expected to so profoundly disrupt embryonic development that it would be unlikely that homozygous or heterozygous germline absence of these moieties will result in vascular malformations in viable organisms.

At first sight similar considerations might apply to VEGF-R3 (Flt4) which binds VEGF-C and VEGF-D, as evidenced by embryonic death of null mice at day E9.5. However, heterozygotes are viable, and in man autosomal dominant inheritance of inactivating mutations in VEGF-R3 cause congenital lymphedema (Irrthum *et al.,* 2000; Karkkainen *et al.,* 2000). This reflects the later role of VEGFR3 in lymphatic vessels to which VEGF-R3 expression becomes restricted, and highlights the concept that heterozygous loss of protein function may indicate functional roles obscured by earlier embryonic lethal events in null mice.

3. Malformations arising from aberrant Tie 2/angiopoietin signaling

3.1 Venous malformations and blue rubber bleb nevus syndrome/ bean syndrome

Bean syndrome, otherwise known as blue rubber bleb nevus syndrome, or VMCM (for venous malformations, cutaneous and mucosal) is inherited as an autosomal dominant trait. The venous malformations are classically observed on the skin where their compressible and nipple-like appearance led to the designation of "blue rubber blebs". Other sites may be affected including the gastrointestinal tract and intracerebral locations. Linkage to chromosome 9 (Boon *et al.,* 1994) led to the identification of causative mutations in the gene encoding the endothelial cell receptor tyrosine kinase Tie-2 (Vikkula *et al.,* 1996).

Tie-2 and its ligand angiopoietin (Ang)1 play crucial roles in late vascular morphogenesis when Tie-2 expression is upregulated (Drake and Fleming, 2000). Mice null for either Tie-2 and Ang1 develop a normal primary vasculature, but display defects in subsequent vascular maturation leading to embryonic lethality, associated with disruption of the assembly of the primitive capillary retiform plexus (Sato *et al.,* 1995; Suri *et al.,* 1996). This does not appear to depend on loss of any mitogenic activity as such activity has not been demonstrated for Ang1. Instead, the abnormalities appear to result from loss of vessel stabilization conferred by Ang1, as evidenced by Ang1 transgenic mice (Thurston *et al.,* 1999). Recent evidence suggests this stabilization is mediated at least in part by regulation of Akt/survivin anti-apoptotic pathways to allow endothelial cells to remain viable during disruption of key intercellular and cellular-matrix communications during angiogenic remodeling (Davis *et al.,* 1996; Pappetropoulos *et al.,* 1999). Ang1 signaling through Tie-2 has a naturally occurring antagonist. Ang2 is a further Tie-2-binding angiopoietin, specifically detected at sites of vascular remodeling, that antagonizes Ang1/Tie2 signaling in endothelial cells (Maisonpierre *et al.,* 1997). Interestingly, Ang2 can act as a Tie-2 agonist in nonendothelial cell lines in which Tie-2 is ectopically expressed (Maisonpierre *et al.,* 1997). As Ang2 mRNA may be increased by hypoxia, VEGF and bFGF, and decreased by Ang1 and TGF-β1, interplay between angiogenic and stability factors is suggested (Mandriota and Pepper 1998).

In disparate Bean syndrome families only two different Tie-2 mutations have been described—C2545T and A2690C leading to missense substitutions Arg849Trp and Tyr897Ser. Each results in a constitutively active kinase, increasing auto-and substrate-induced phosphorylation (Vikkula *et al.,* 1996; Calvert *et al.,* 1999). The mutant Arg849Trp receptor is also able to activate additional intracellular signaling pathways as demonstrated by the activation of STAT1 in addition to STAT3 and STAT5 (Korpelainen *et al.,* 1999). It is not clear whether the constitutively active Tie-2 protein simulates predominantly Ang2 or Ang1 signaling. These results would predict inappropriate regulation of endothelial cell apoptosis in the generation of the vascular lesions in Bean syndrome, but direct data is awaited.

4. Malformations resulting from transforming growth factor (TGF)-β superfamily dysfunction

The TGF-β superfamily including TGF-βs, activins, bone morphogenetic proteins (BMPs), growth/differentiation factors (GDFs) and inhibins signal through heteromeric complexes comprised of type I and type II cell surface receptor serine-threonine kinases (Heldin *et al.,* 1997; Massague and Chen 2000; Miyazono *et al.,* 2001; Wrana *et al.,* 1994). Activated type I receptors phosphorylate cytoplasmic receptor-associated Smad proteins (R-Smads) which oligomerize with a co-Smad molecule, Smad4, and translocate to the nucleus to act as transcription factors and alter gene expression. The intriguingly limited R-Smad repertoire separates ligand/receptor groups into two groups, based on whether AR-Smads (Smad2 and SmadB, classically TGF-β and activin R-Smads) or BRSmads (Smadl, SmadS and SmadS, typically BMP and GDF R-Smads) are utilized. The

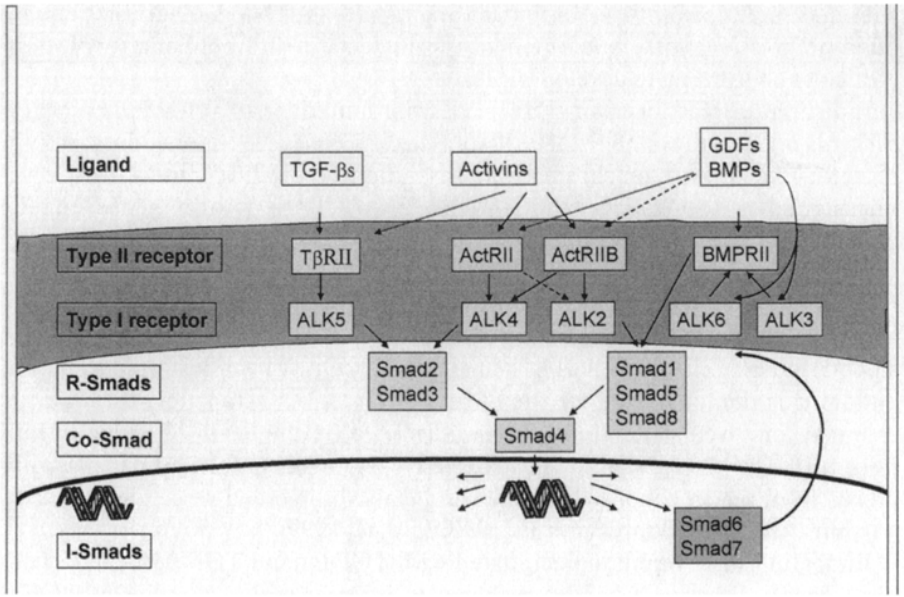

Figure 1. TGF-β signaling pathways. Cartoon illustration of simplistic pathways involved in signaling by TGF-β superfamily members (see text for details).

inhibitory I-Smads, Smad6 and Smad7, antagonize these pathways in negative feedback loops (Afrakhte *et al.,* 1998; Itoh *et al.,* 1998; Takase *et al.,* 1998). Smad7 acts as a general inhibitor of TGF-β family signaling (Ishisaki *et al.,* 1999; Souchelnytskyi *et al.,* 1998), whereas Smad6 which is induced by Smad1/5 signaling (Ishida *et al.,* 2000) appears to preferentially inhibit BMP signaling (Ishisaki *et al.,* 1999; Itoh *et al.,* 2001). *Figure 1* provides an overview of our current understanding of these pathways.

These ligands, their receptors and downstream signaling moieties regulate a diverse series of fundamental pathways in development and pathophysiology. The importance of the TGF-β pathway proteins in the development and maintenance of the vasculature has been repeatedly demonstrated by null mouse models. Similar abnormalities in yolk sac vascular development is observed amongst the plethora of additional and varying embryonic lethal phenotypes in mice deficient inTGF-β1 (Dickson *et al.,* 1995) the TGF-β receptors TβII (Oshima et al., 1996) and TβRI (Larsson *et al.,* 2001), SmadS (Chang *et al.,* 1999) and Smad6 (Galvin *et al.,* 2000). Perturbation of these pathways by genetic defects is now known to be responsible for several vascular diseases.

4.1
Arteriovenous malformations and hereditary hemorrhagic telangiectasia

The autosomal dominant vascular disease hereditary haemorrhagic telangiectasia (HHT, also known as Osler-Weber-Rendu syndrome) was the first to be ascribed to aberrant TGF-β superfamily signaling, although the exact pathogenetic mechanisms remain obscure. HHT is characterized by mucocutaneous telangiectasia derived from post-capillary venules (the majority exhibiting direct arteriovenous communications), and larger arteriovenous malformations (AVMs) in the lungs, brain, liver, and spine (Shovlin *et al.,* 1999). In contrast to cerebral AVMs that are thought to develop perinatally, the overwhelming majority of abnormal vessels in HHT develop postnatally. As a result, HHT exhibits age-dependent penetrance, with most index patients presenting in early to mid-adulthood. Manifestations are often progressive, with mucocutaneous telangiectasia and pulmonary AVMs in particular continuing to develop with time.

Indistinguishable forms of HHT arise from mutations in at least three genes: *endoglin* on chromosome 9 (McAllister *et al.,* 1994), *ALK-1* encoding activin receptor-like kinase I, on chromosome 12 (Johnson *et al.,* 1996), and a third as yet unassigned gene (Wallace and Shovlin 2000). Both known genes encode endothelial-cell expressed proteins involved in TGF-β superfamily signaling.

Endoglin and HHT type I. The transmembrane glycoprotein endoglin is widely expressed in the vasculature, being detectable in both endothelial and vascular smooth muscle cells, as well as in hematopoietic cells. The crucial importance of endoglin is demonstrated by the effects of endoglin deficiency. Endoglin is essential for vascular development as illustrated by the death of endoglin null mice at 10–10.5 days gestation due to defective remodeling of the primary vascular plexus resulting in abnormal yolk sac and embryonic blood vessel development (Arthur *et al.,* 2000; Bourdeau *et al.,*1999; Li *et al.,* 1999).

Endoglin can be immunoprecipitated with TGF-β1 and TGF-β3, but does not bind ligand directly. It has been shown to interact with a number of TGF-p ligands through interactions with the respective ligand-binding receptors T(3RII (for TGF-β1, TGF-β3), ActRII (activin A, BMP-7) and ALK-3 and ALK-6 (BMP2) (Pece *et al.,* 1999). Although its function is not completely clear, endoglin expression in cultured cells has been shown to inhibit a subset of cellular responses to TGF-β1 in a variety of assays, including TGF-fU-induced inhibition of cellular proliferation by down-regulation of *c-myc,* upregulation of PECAM activation by phosphorylation (Lastres *et al.,* 1996), and upregulation of the plasminogen activator inhibitor (PAI)-l promoter (Letamandia *et al.,* 1998). In contrast, other TGF-β1-induced responses including the feedback loops increasing mRNAs for TGF-β1 and its signaling receptors TβRI and TβRII, and TGF-β1 modulation of integrin levels are unchanged (Lastres *et al.,* 1996). In part this is possible since stable overexpression of endoglin, in spite of altering PAI-1 promoter and *c-myc*- transcriptional responses to TGF-β1 administration in myoblasts, does not alter transcriptional activity of the Smad7 promoter thus preventing blanket downregulation of the system (Begbie and Shovlin, unpublished).

Over 50 different endoglin mutations have been described to date in HHT (Abdalla *et al.,* 2000; Cymerman *et al.,* 2000; Gallione *et al.,* 1998, 2000; McAllister *et al.,* 1994, 1995; Paquet *et al.,* 2001; Pece *et al.,* 1997, 1999; Shovlin *et al.,* 1997; Yamaguchi *et al.,* 1997) comprising in-and out-of-frame deletions, splice site and missense mutations. The prevailing view is that the molecular mechanism of HHT development is via endoglin haploinsufficiency, rather than through a dominantnegative interaction. Evidence for this includes the following considerations: 1) In all cases of HHT which have been characterized at the level of the endothelial cell, endoglin surface expression is half that of normal (Bourdeau *et al.,* 2000). 2) Pulsechase analysis performed in native HUVEC cells derived from the umbilical cords of babies with confirmed endoglin mutations has shown that many deletion mutant proteins are either not detectable intracellularly, or are only detected as a transient species. Many missense mutants were expressed in an intracellular precursor form, but were not properly processed and did not reach the cell surface (Paquet *et al.,* 2001; Pece *et al.,* 1997). 3) There is no detectable clinical difference in phenotype between HHT patients with null (absent mRNA) mutations, inframe or out-of-frame deletions (Shovlin *et al.,* 1997). 4) The human HHT phenotype can be recapitulated in endoglin$^{+/-}$ heterozygous mice which in one series, with time develop signs of HHT, including visible telangiectasia and bleeding from the ears, skin, nose, and genitals, and visceral vascular malformations (Bourdeau *et al.,* 1999). Debate continues whether there may be occasional examples of mutations acting in a dominant negative manner (Lux *et al.,* 2000; Paquet *et al.,* 2001). The most severe HHT phenotypes are seen in patients with proven null mutations (Shovlin *et al.,* 1997), indicating that any dominant negative mutants would be unlikely to contribute to a significant proportion of HHT cases. Nevertheless, we have demonstrated functional reversal of the effects of endoglin transfection by overexpression of the extracellular domain of endoglin in a series of stably expressed truncated and phosphorylation mutant endoglin peptides (Begbie and Shovlin, manuscript in preparation). As these could be generated *in vivo* by single nucleotide changes, this highlights the possibility that there may be occasional naturally occurring dominant negative mutants.

ALK-1 and HHT type 2. Were it not for the identification of *ALK-1* mutations resulting in HHT, there might be further debate as to whether perturbation of one of the documented TGF-β1 responses or other as yet uncharacterized endoglin functions is critical in generating the HHT lesions. *ALK-1* encodes the TGF-β superfamily type I receptor activin-like receptor kinase (ALK-1) which is expressed almost exclusively in endothelial cells. The role of ALK-1 within the superfamily remains unclear. It can bind TGF-β1, TGF-β3 (Lux *et al.,* 1999) and TβRII (Oh *et al.,* 2000), functionally inhibits TGF-β1 signaling through TβR1/ALK-5, and may act in determining a critical balance of TpRI/ALK-5 and ALK-1 for TGF-β1 signaling (Oh *et al.,* 2000). However, in P19 cells, constitutively active ALK-1 predominantly activates BR-Smad pathways usually associated with BMP signaling (Macias-Silva *et al.,* 1998).

Numerous *ALK-1* mutations have been described to date (Johnson *et al.,* 1996; Abdalla *et al.,* 2000; Berg *et al.,* 1997; Kjeldsen *et al.,* 2001). Again, haploinsufficiency is thought to be the molecular cause of disease since in all cases of HHT which have been

characterized at the level of the endothelial cell, ALK-1 surface expression is half that of normal (Abdalla *et al.,* 2000).

Direct interactions between endoglin and ALK-1 are predicted in view of the HHT link, and in the absence of ligand, endoglin can be immunoprecipitated with a polyclonal αALK-1 antibody from human umbilical vein endothelial cells suggesting an interaction between endoglin and ALK-1 (Abdalla *et al.,* 2000). There are similarities in defects in null mice: ALK-1 homozygous knockout mice also die midgestation due to vascular abnormalities (Oh *et al.,* 2000; Urness *et al.,* 2000). However, in these mice, excessively large vessels arising from aberrant capillary fusion (Oh *et al.,* 2000), or aberrant arteriovenous shunting (Urness *et al.,* 2000) are observed. In one study this was associated with the loss of expression of Efnb2, the earliest marker defining arterial identity (Urness *et al.,* 2000). These subtle differences in the vascular phenotypes predict that endoglin-ALK-1 interactions may not be required for all functions of either molecule.

Triggers for vascular defects in HHT. As in other vascular malformation syndromes, in any one individual with HHT, however severe, the majority of vascular beds develop normally with macroscopic malformations developing in only a small fraction. Similar to many other autosomal dominant diseases, different family members with the same underlying mutation in *endoglin* or *ALK1* have highly disparate phenotypes, which in the case of HHT may range from life-threatening events in childhood to non-penetrance. To what extent are environmental or genetic factors responsible?

Physiological factors including changes in hemodynamics and hormones are likely to play a role; pulmonary AVMs are more common in women than in men with HHT, and have been shown to enlarge during pregnancy, with increased risk of pulmonary hemorrhage and death (Shovlin *et al.,* 1995). However, the heterozygous null mouse emphasizes the importance of modifier genes on disease expression. When the endoglin mutant allele was bred into a 129/Ola background, significantly more heterozygous mice showed disease signs (72%) than with the mutant allele bred into a C57BL6 background (7%) (Bourdeau *et al.,* 2001). Intercrosses showed an intermediate incidence of disease. This same report describes differences in plasma TGF-β1 levels between these mice strains with levels significantly lower in the 129/Ola strain. Loss of one functioning endoglin allele also was associated with lower circulating and latent TGF-β1 levels, suggesting that decreased plasma levels of TGF-β may contribute to the clinical variability seen in the human disease. Additional modifying influences are predicted, and may be defined from analyses of other vascular disorders of TGF-β signaling.

4.2
Pulmonary arterial abnormalities in primary pulmonary hypertension (PPH)

One such disease may prove to be primary pulmonary hypertension (PPH). While not strictly a vascular malformation syndrome, this vascular disease inherited in some families as an autosomal dominant trait does lead to progressive muscularization of the walls of small pulmonary arteries and associated plexiform lesions of proliferating endothelial cells. Ultimately, increased pulmonary vascular resistance leads to right heart failure, and

without heart-lung transplant, usually leads to death within a few years of diagnosis. The full-blown syndrome has an incidence of 2–3 per million people per year. Females are more commonly affected than men, with a ratio of about 2 to 1, and presentation is usually in the mid-thirties.

Studies on the approximately 6% of cases with an obvious autosomal dominant, partially penetrant pattern of inheritance identified a PPH locus on chromosome 2. In 2000, two groups identified heterozygous germline mutations in *BMPR2* encoding bone morphogenetic protein receptor, BMPR-II (Deng *et al.,* 2000; Lane *et al.,* 2000).

BMPR-II allows signaling by a number of TGF-β superfamily ligands, initial evidence suggesting this would predominantly involve the type I receptors ALK-3 and ALK-6, though association with other type I receptors, particularly those such as ALK-1 and ALK-2 which signal through BR-Smads seems likely. A vascular role for BMPR-II was not evident from null mice as these died before the crucial stages of vasculogenesis in which other TGF-β superfamily members were implicated, due to defective epiblast and mesoderm differentiation (Beppu *et al.,* 2000).

As with HHT, the mechanism of PPH resulting from *BMPR2* mutations appears likely to be haploinsufficiency; no productive dominant-negative mutants have yet been identified (Machado *et al.,* 2001). At least 25% of apparently sporadic cases of PPH have also been shown to result from *BMPR2* mutations, suggesting that the incidence of familial cases is likely to be grossly underestimated (Thomson *et al.,* 2001).

Although the molecular basis of PPH is understood, there again remain many questions, including why the systemic circulation is spared, and why particular pulmonary arteries develop abnormally after often many years of macroscopic and physiological normality. As in HHT, significant heterogeneity in phenotype is observed associated with the same mutation, suggesting additional environmental or genetic factors are required for the full clinical phenotype to develop (Machado *et al., 2001*). Clinical associations suggest that use of anorexigens such as aminorex, fenfluramine and dexfenfluramine, toxic rapeseed oil, amphetamines, L-tryptophan and HIV infection may provoke the disease in genetically susceptible individuals (Rich, 1998). One tantalizing explanation may be the occurrence of somatic second hits, analogous to the observations of Knudsen in cancer cells. In PPH, monoclonal proliferations of endothelial cells occur in plexiform lesions: These display somatic genetic instability as evidenced by mutations in microsatellite repeats, *TβRII,* and in patients exposed to triggering anorexigens, *Bax* (Yeager *et al.,* 2001). Further studies are underway to determine if reduced TpRII activity (undetectable in 90% of the plexiform lesions (Yeager *et al.,* 2001)) or other somatic mutations have impaired the function of BMPRII protein encoded by the germ-line wildtype allele of *BMPR2.*

4.3
The overlap between PPH and HHT

The differences between these two diseases, particularly in the pulmonary circulation, the sole site of the obstructive PPH lesions, and the site of dilated lesions in 30–50% of HHT patients, at first sight appear overwhelming. A recent study, however, has demonstrated

that PPH indistinguishable to that seen in classical PPH families with *BMPR2* mutations is observed in a proportion of type II HHT patients with *ALK-1* mutations, but normal *BMPR2* sequence (Trembath *et al.,* 2001). Intriguingly, PAVMs are less common in *ALK-1* than *endoglin* HHT families (Berg *et al.,* 1996). This suggests that dysregulation of the vascular signaling pathways which produce the vascular dilations characteristic of HHT also can lead to the over-proliferation and closure of the small vessels of the lung seen in PPH, and increases speculation that ALK-1 may function in BMP rather than TGF-β1 signaling pathways in terms of pathogenesis of these vascular disorders.

5.
Defective cytoplasmic signaling

In contrast to the previously described syndromes in which aberrant responses to extracellular signals appear causative, evidence is accumulating that a number of vascular malformation syndromes arise as a result of dysregulated intracellular signaling. Particularly prominent are mutations in proteins which have been thought to participate in key intracellular switches between activated and inactivated members of the Ras family. However, recent data is highlighting that this may oversimplify pathogenic processes to such a degree as to mask the critical pathogenetic events.

5.1
Capillary venous malformations (cerebral cavernous angiomas/ malformations, CCM): defective ras or integrin signaling?

Cerebral cavernous angiomas or malformations (CCMs) consist of contiguous and isolated endothelial cell channels, derived from the venous side of the circulation. There is little flow through the lesions, and CCMs contain organizing thrombus. Morphological characterization of CCMs by electron microscopy has demonstrated that these malformations contain virtually exclusively endothelial cells, on an often multilaminar basal lamina that is surrounded by an exceptionally thick collagenous matrix. Endothelial cell apposition appears defective: classical tight junctions were not observed, and at some sites between the endothelial cells, basal lamina was exposed directly to the sinusoidal lumen with evidence of hemorrhage through these sites (Clatterbuck *et al.,* 2001). Such features would imply that these weakened vascular walls would predispose to subtle hemorrhage that may account for a less stable structure, as indicated by the frequent incidence of epileptic seizures in affected patients.

Familial forms of CCMs with autosomal dominant inheritance enabled linkage studies to map three loci in different families to chromosomes 7q (CCM1) (Gunel *et al.,* 1995), 7p (CCM2) and 3q (CCM3) (Craig *et al.,* 1998). CCM1 appears to be involved in CCM in the majority of families of Mexican or Hispanic origin where a founder effect operates (Gunel *et al.,* 1996), whereas the other loci were first described in Caucasian kindreds.

The CCM1 gene has now been identified as encoding Krev interaction trapped 1 *(Krit1)* (Couteulx *et al.,* 1999; Sahoo *et al.,* 1999), first identified in a yeast twohybrid screen as an interactor with RAP1A/Krev-1. Early mutations described in *Krit1* included missense

mutations and frameshifts predicted to lead to stop codons between nucleotides 206 and 1283, and a splice site mutation leading to an inframe deletion of the RAPl A interacting region in exon 11. It was predicted that Kritl mutations would affect intracellular signaling pathways involving Ras GTPases since RAPl A/Krev-1 was thought to act as a ras antagonist (Serebriiskii, 1997). However, it is now apparent that there are additional coding sequences 5' to the sequences analyzed in early mutational studies, extending the protein from the originally described Krit1 of 529 aminoacids which interacts with RAP1A/Krev-1, to a full length 736 amino acid moiety which does not (Zhang *et al.,* 2001). Instead, the full length moiety interacts with integrin cytoplasmic domain-associated protein-1 (icap1α), highlighting a potential role for the wildtype proteins in integrin-mediated signaling, and proving a putative role for the four ankyrin repeat domains in the originally described protein as these are often observed in integrin-associating proteins, modulating cytoskeletal links (Zhang *et al.,* 2001).

Although there is no direct data implicating the mutant Krit1 proteins in either ras or integrin-mediated events, the electron microscopy data indicating abnormal endothelial cell junctions in the lesions, might support the latter. Alternatively, signaling pathways that control physical interactions between endothelial cells might be deranged indirectly as a consequence of the primary pathological events leading to the formation of the abnormal endothelial cell cluster.

5.2
Hyperkeratotic cutaneous capillary-venous malformations (HCCVMs)

Cutaneous capillary-venous malformations at other sites also result from *Krit1* mutations. In hyperkeratotic cutaneous capillary-venous malformations (HCCVMs), dilated cutaneous capillary and venous channels lie in the dermis and hypodermis, beneath a hyperkeratotic epidermis. These lesions occur in a subgroup of CCM families and all individuals within these families with HCCVMs also have cerebral manifestations. In one CCM/HCCVM family, a single nucleotide deletion in exon 1 (ΔG103) predicting a truncation early in the protein was described (Eerola *et al.,* 2000), with rt-PCR evidence for reduced mutant mRNA stability As the mutation described in this report truncates the KRIT1 protein earlier than the other mutations identified as causing CCMs, the authors proposed that the cerebral manifestations may result from a KRIT1 protein with some residual function, while the HCCVMs might require more complete loss of function. Whether this relates to differing influences on the proposed modulation of integrin and ras pathways awaits further study.

5.3
Arterial malformations in neurofibromatosis type I

Neurofibromatosis is classically recognized by the presence of cutaneous, patchy hyperpigmentation and the development of benign and malignant tumors from neural crest cells. In type I neurofibromatosis, perhaps 10% or more of individuals have

abnormal arteries displaying particularly accumulations of abnormally proliferating smooth muscle cells within the intima, resulting in significant morbidity due to renal artery stenosis leading to hypertension, and occluded arteries leading to infarcts (Riccardi, 1992; Salyer and Salyer, 1974). Arteriovenous malformations and aneurysms are also observed. These processes are usually distinct from any adjacent neurofibromas, and appear to arise from within the artery itself.

NF1 results from mutations in *neurofibromin* on chromosome 17 (Xu *et al.,* 1990) encoding an intracellular protein. Neurofibromin acts as a tumor suppressor as the central region of neurofibromin displays sequence similarities with GTPase activating proteins (GAPs) which downregulate members of the ras family (Weiss *et al.,* 1999) from GTP-bearing (active) to GDP-bearing (inactive) states, reducing mitogenic signaling. NF-associated tumors frequently display somatic loss of the second NF allele, increased ras activity and increased cellular proliferation (Gutman *et al.,* 2001): normal growth and cytokine signaling can be restored in $NF^{-/-}$ cells by over-expression of the NF GAP-related domain (Hiatt *et al.,* 2001).

Vascular involvement in NFl is not so readily explained in these terms. Evidence for abnormal mitogenic activity may be provided by the abnormal accumulations of intimal smooth muscle cells observed in NF1 vascular lesions, and two of the mammalian Ras proteins, *neurofibromin* and p120-rasGAP which act synergistically in embryonic vascular development, at a stage of reorganization of the early yolk sac vascular plexus, and embryonic dorsal aorta (Henkemeyer *et al.,* 1995). However, somatic loss of a second allele to further perturb cell signaling pathways mediating the proliferation response to growth factors would need to occur at a high frequency in patients with extensive disease. It is increasingly recognized that there are significant other differences in $NF1^{-/-}$ cells that may potentiate aberrant vascular development: for example, $NF1^{-/-}$ Schwann cells aberrantly express the angiogenic factor midkine (Mashour *et al.,* 2001) which *in vitro* acts as an endothelial cell mitogen, and $NF^{+/-}$ Mast cells which release several proangiogenic factors are hyperproliferative (Ingram *et al.,* 2001). Recent hypotheses have however focussed on the possibility that haploinsufficiency of neurofibromin provokes an aberrant response to local trauma. Since the arterial distribution of lesions resembles that of atherosclerosis, aberrant responses to flow-and shear-stress induced endothelial cell injury are currently favored as possible triggers (Hamilton and Friedman, 2000), and importantly, suggest possible therapeutic strategies through scrupulous antihypertensive regimes. Again, the precise nature of such an aberrant repair process, and whether this is solely or in any way resultant upon aberrant ras signaling is not clear.

6. Overlaps and perspectives

Although these diseases are discussed separately, emerging evidence is beginning to link the various systems disrupted in their pathogenesis. For example, Ang2 mRNA levels are decreased by exposure to TGF-β1 (Mandriota and Pepper, 1998), and sporadic brain arteriovenous malformations similar to those observed in HHT display decreased angiopoietin-1 and increased levels of angiopoietin-2 (Hashimoto *et al.,* 2001). It has also

been proposed that some of the numerous and complex intracellular roles of Ras proteins may be to operate downstream of endothelial cell specific receptors including Tie2, mutated in venous malformations (Henkemeyer *et al.,* 1995).

Identification of genes mutated in further diseases, particularly autosomal recessive states less readily identified should help to clarify some of these issues. For instance, identification of the third HHT gene (Wallace and Shovlin, 2000), and the gene mutated on chromosome 1 giving rise to venous malformation with glomus cells (VM-GLOM) (Boon *et al.,* 1999; Brouillard *et al.,* 2000) are eagerly awaited.

The importance of the vascular processes identified by studying these rare diseases is likely to become more evident as we understand more regarding the finer regulatory interactions and physiological and pharmacological mechanisms to modulate these signaling pathways abruptly perturbed by genetic mutations in experiments of nature.

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10
Comparison of genetic programs for embryonic vascular development and adult angiogenesis

J.Douglas Coffin

1. Introduction

The task of comparing genetic programs for neovascularization between an embryo or fetus and an adult is fundamentally a question of how the genome participates in vascular development; then how the genome functions in the adult for vascular homeostasis or pathologies that activate angiogenesis. Morphogenesis of the vascular system involves several fundamental molecular and cellular processes such as differentiation, proliferation, migration, determination, adhesion and apoptosis *(Figure 1)*. All of these processes are at play throughout developmental and adult stages because they are interwoven in any fundamental genetic program for tissue growth and renewal.

The genetic program for a given process, i.e. vascular development or vascular homeostasis, is a subset of gene expression from the entire genome *(Figure 2)*. Defining a genetic program for vascular development is subsequent to defining the overall developmental genetic program. The latter is represented by that subset of the genome expressed during development, and the former represents gene expression specifically associated with vascular development. Likewise, the genetic program for adult vascular homeostasis represents the subset of genes derived from the adult genetic program.

Development is a multidimensional process leading to anatomical regions and physiological specializations. There are three geometrical dimensions (x, y, and z), with time as a fourth dimension. Other dimensions are represented by the molecular and cellular processes described above: proliferation or quiescence, various stages of differentiation or apoptosis, and adhesion or active migration. Trying to

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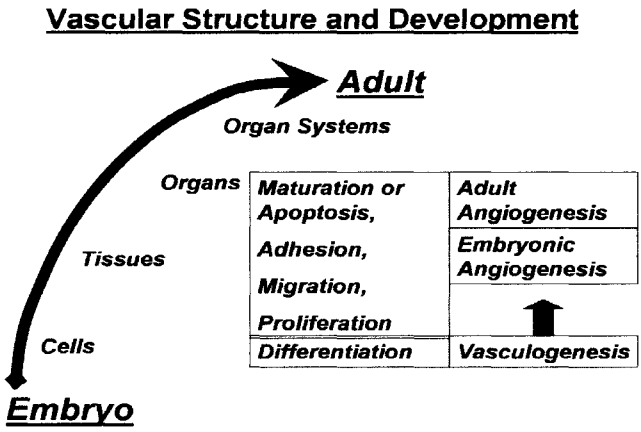

Figure 1. Vascular structure and morphogenesis. Differential gene expression forms the basis for morphogenesis of the circulatory system, shown as a continuous process from cells to tissues to organs and organ system; the circulatory system. The inset box shows the role of vasculogenesis, embryonic angiogenesis and adult angiogenesis in this scheme. Vascular tissue develops early through vasculogenesis then embryonic angiogenesis. As various tissues, organs and organ systems develop, the vascular system is an inherent, vital tissue that is interwoven through other tissues and organs by embryonic angiogenesis. In the adult, the vascular system is maintained by homeostatic, adult angiogenesis.

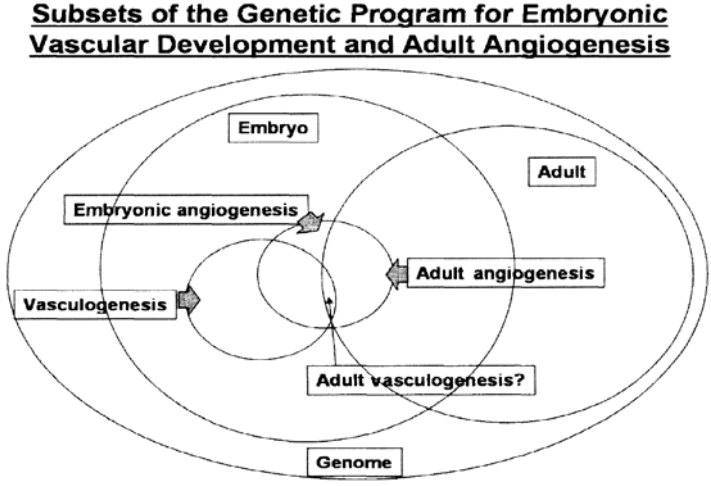

Figure 2. Gene expression. A venn diagram of gene expression in the adult and embryonic stages of vasculogenesis and angiogenesis. The genome encompasses all gene expression, with adult and embryonic subsets that intersect with one another. Then embryonic and adult angiogenesis are both included in the embryonic subset of genes, because adult angiogenesis was derived from embryonic angiogenesis, and likewise for both embryonic and adult vasculogenesis.

characterize a given cell, protein or gene in a specific dimension speaks to the complexity of development as a discipline. This principle certainly applies to vascular development, while adult vascular homeostasis represents a simpler system because it has fewer dimensions. One of the first specializations in vascular development is formation of large and small blood vessels or macro-and microvasculature. Establishment of the dorsal aortae, umbilical and vitelline vessels in midgastrulation is an example of large versus microvascular specialization early in development. Then further development of various microvasculatures in specific regions of the embryo, the brain or kidney for example, represent further specializations. Remodeling the embryonic vessels into definitive adult structures is yet another specialization.

It is well established that the microvasculature in an adult is not homogeneous (Thorin and Shreeve, 1998). Rather, the microvasculature is defined by location and association with other tissues and its lineage. For example, brain microvasculature with a blood-brain barrier has considerably different characteristics than the microvessels in the kidney with glomerular filtration. Cell determination and differentiation to this end can result from induction by association with brain or kidney tissues during vascular morphogenesis, and from the origin of the precursor cells and vessels during development. The vessels in an adult organ may originate from several different embryonic tissues that have long since been remodeled. But those origins can contribute to the phenotype of the adult vasculature. The genetic program must, therefore, incorporate gene expression and subsequent induction between multiple tissues, and it must include fate maps of the cells in terminal differentiation.

The logical beginning for studying the vascular development genetic program is the point when cells are first fated for the vasculature. For purposes of simplicity, we will begin with presomite stages in gastrulation, where the earliest lineage maps have effectively traced the lineage for the definitive embryonic vasculature (Evans, 1909). The end-point might be a representative "adult" stage. From the perspective of a developmental biologist, aging in an adult can be perceived as an extension and transition of developmental processes observed in the embryo. Thus, an organism never stops "developing" throughout its existence. Aging may result from degeneration of the developmental programs by depletion of stem cells, damage to the organism that is outside the domain of tissue renewal and other sources for depletion of tissue renewal capabilities. Thus, defining a representative stage for comparison between an embryo and an adult is arbitrary because we are only choosing a stage in the continuum of development and aging.

Therefore, this comparison of vascular-specific genetic programs, for the embryo and the adult in homeostasis and pathology, will constitute the functional interaction of the genome during multidimensional changes in the vasculature, all in the context of the developmental and aging processes described above. The spatial dimensions are represented by regions of the embryo, embryonic tissues structures and organs, then adult tissues, structures, and organs. The temporal dimension is represented by stages of development and aging. The cell determination and differentiation dimensions are represented by endothelial cell phenotypes and corresponding vascular gene expression patterns.

2.
The genetic program for embryonic vascular development

Vascular development in the embryo has been studied for decades. Eloquent descriptions at the beginning of the twentieth century (Evans, 1909; Sabin, 1917, 1920) served as the basis for more refined descriptions from modern use of immunocytochemistry, cell culture, and molecular genetics (Coffin and Poole, 1988; Pardanaud *et al.,* 1987, 1989). A greater understanding of cell biology has resulted in comprehensive models for vascular morphogenesis that include cell proliferation, migration, determination, differentiation, adhesion and apoptosis (Risau, 1997). Genomic and differential gene expression data now provide for an integrated genetic model whereby these cellular processes are orchestrated by genetic programs in concert by queues from the various regions of the embryo.

Most discussions of embryonic vascular development are based on descriptions of *de novo* formation of vascular anlagen and subsequent vascular sprouting (Coffin and Poole, 1988; Poole *et al.,* 2001). The classical descriptions included these events (Sabin, 1917, 1920), but modern descriptions placed them in the context of functional cell and developmental biology complete with molecular and genetic regulatory schemes (Pardanaud and Dieterlen-Lievre, 2000). Differentiation of ES cells *in vitro* produced the first allusion to *de novo* vascular development as *vasculogenesis* and subsequent vascular sprouting as *angiogenesis* shown in *Figure 3* (Doetschman *et al.,* 1985, 1993). These terms were then applied *in vivo* to developing avian embryos (Pardanaud *et al.,* 1989; Poole and Coffin, 1989) and functionally delineated as distinct developmental processes by use of quail/chick chimeras. Later descriptions and use of gene targeting have shown evolutionary conservation of vasculogenesis and angiogenesis during vascular development from birds to mammals (Coffin *et al.,* 1991; Yancopoulos *et al.,* 2000). This analysis begs the question of whether the processes and regulatory schemes for embryonic angiogenesis are conserved in the adult for vascular homeostasis and whether the embryonic mechanisms are activated in pathologies that stimulate angiogenesis.

2.1
Vasculogenesis

The genetic program for embryonic vascular development logically begins with the earliest process: Vasculogenesis, i.e. *de novo* differentiation of angioblasts, or free endothelial precursors, from mesoderm, then cell migration and subsequent cell-cell and cell-substrate adhesion to form vascular chords *(Figure 3).* QH-1 staining of quail embryos reveals the first evidence of vasculogenesis coincident with formation of the first somites. There is extraembryonic vasculogenesis on the yolk sac and it has remained a mystery whether there is any connection between extraembryonic vasculogenesis and intraembryonic vasculogenesis. A similar debate continues over the origins of hematopoietic stem cells. Quail/chick chimeras and explants have shown that *de novo* differentiation of angioblasts is possible in the absence of the yolk sac and the fate maps have shown that the precursors for the embryonic vessels arise from embryonic mesoderm. This does not, however, discount the possibility that yolk sac cells may contribute to the

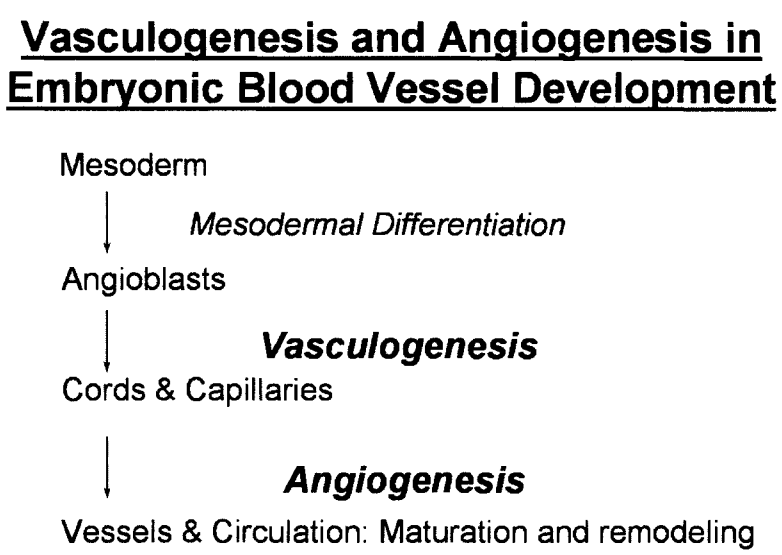

Figure 3. Vasculogenesis and angiogenesis. The genetic program drives the accepted concepts for the two principal modes of embryonic blood vessel development. Vasculogenesis involves *de novo* formation of the endothelium from mesodermal derivatives *in situ;* a result of mesodermal differentiation. The cords and capillaries resulting from vasculogenesis are then expanded and remodeled through embryonic angiogenesis to form the functional embryonic circulatory system; the first functional organ system in the embryo.

embryonic vasculature. The subset of genes that function in mesodermal-angioblast differentiation is not well defined because no nullmutation or specific inhibitor has been identified that blocks this stage of vasculogenesis. However, gain of function experiments have shown that VEGF and FGF-2 can enhance this process (Cox and Poole, 1991; Drake and Little, 1995). Growth factors, including FGF-2, have also been implicated in mesodermal differentiation (Skaer, 1997) and targeted deletion of FGF receptors results in embryonic lethality early in development prohibiting mesodermal differentiation altogether (Arman *et al.,* 1998; Deng *et al.,* 1994). Thus, the angiogenic growth factors enter the genetic program early in vasculogenesis. Targeted deletion of the receptor tyrosine kinase (RTK) flk-1 results in arrest of vasculogenesis during the angioblast differentiation stage (Shalaby *et al.,* 1995) while deletion of the RTK flt-1 causes death from edema following initial formation of definitive blood vessels (Fong *et al.,* 1995). Both of these RTKs are VEGF receptors but they appear to affect embryonic vascular development in different ways. Deletion of flk-1 causes an arrest in vasculogenesis while deletion of flt-1 causes a physiological malfunction of patent vessels.

The gene targeting results for loss of function and *in vitro* gain of function data reveal functional nodes in vasculogenesis where malfunctions in the genetic program specifically affect vascular development. The first node is mesodermal-angioblast differentiation and subsequent proliferation, then the next node is cell-cell adhesion and lumen formation to

form a functional, intact monolayer. It is curious that two different VEGF receptors differentially perform a cell differentiation/proliferation function (flk-1) and a vascular permeability function (flt-1). However, the data from adult, pathological angiogenesis suggests that this dual VEGF function is conserved in the adult. Conversely, we may state that the adult program for VEGF-related pathological angiogenesis recapitulates the embryonic program.

Implicit in growth factor function are related cellular processes regulated by growth factors including signal transduction, cell cycle regulation, cell adhesion and apoptosis. To date there are little functional data to suggest that deletion genes for signal transduction, cell cycle regulation, cell migration or apoptosis specifically affect the endothelium or vasculogenesis. It is, therefore, highly likely that similar signaling and regulatory pathways found in adult endothelium and other cells for growth-factor-related signal transduction, cell cycle regulation and apoptosis are at work in the mesoderm, angioblasts, and capillary plexi during vasculogenesis. This suggests a conservation of regulatory genes for these cellular processes in the genetic program between cells and tissues. For example, map kinase mediated signal transduction (Oettgen, 2001), regulation of the cell cycle (Stromblad *et al.,* 1996) and apoptosis (Dimmeler and Zeiher, 2000) are features of endothelial cells that are very similar, but not exactly the same, as several other cell types.

The genetic program for vasculogenesis shows much more specificity in expression and function of cell-cell and cell-substrate adhesion molecules. For example, epithelial cell cadherin (ECAD) is expressed in most epithelium, except the vasculature where Vascular Endothelial Cadherin (VE-CAD) is specifically expressed by the developing endothelium and essential for vasculogenesis (Heimark *et al.,* 1990; Nachtigal *et al.,* 2001). Likewise, the alpha-V beta 3 integrin is essential for cell-substrate adhesion in vasculogenesis (Drake *et al.,* 1995). Differential expression of cadherins (Haselton and Heimark, 1997) and integrin (Friedlander *et al.,* 1995) adhesion molecules is, therefore, essential in the genetic program for vasculogenesis where growth factors and other morphogens can differentially regulate expression of cell adhesion molecules (19). These data are consistent with fluid models for tissue morphogenesis (Steinberg and Takeichi, 1994) and other leukocyte homing models where different cell types, including angioblasts differentiating from mesodermal precursors, are able to sort and differentially adhere to form tissues based on the subset of CAM, CAD and integrins expressed on the cell surface *(Figure 4)*.

The essential difference between the specificity of regulatory molecules for these cellular processes lies in the redundancy and specificity of the genetic program for vascular development. Growth factor data, for example, suggest a high level of specificity in the cell surface growth factor receptors, while temporal and spatial expression of the growth factor proteins has low specificity. There is considerable redundancy in some angiogenic growth factor families such as the FGFs and TGFβs, with much less redundancy in the endothelial-specific growth factors such as the angiopoietins and VEGFs. Close examination of growth factor function reveals a functional hierarchy where broad-spectrum, angiogenic growth factors that are not vascular specific are widely expressed to trigger cell proliferation and activation. This may be envisioned as an early response to tissue morphogenesis, repair and replacement. Examples of broad-spectrum growth factors functioning in this capacity include the FGFs, PDGFs, TGFβs. The next

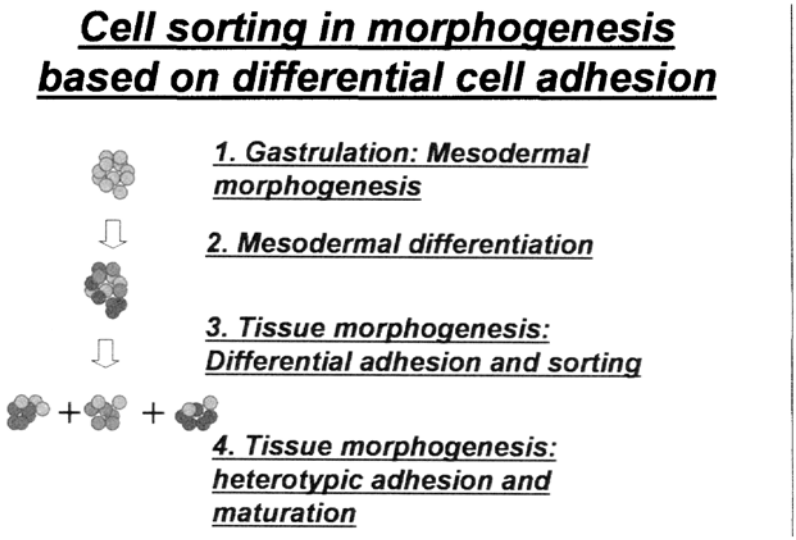

Figure 4. Cell adhesion and cell sorting. Differential gene expression in CAMs is an essential part of the genetic program. Mesodermal differentiation is regulated by "morphogens", principally including growth factors and hormones. The morphogens alter expression of cell adhesion molecules for cell sorting, analogous to separation of different phases of liquids. Differential adhesion results in clustering of like cells, expressing homotypic CAMs, for cell sorting. Conversely, heterotypic CAM expression results in exclusion of cells that are not capable of adhering to one another. Finally, once the tissues have begun maturation, the CAM expression program changes and the various cells and tissues will heterotypically adhere to one another to complete morphogenesis and organogenesis.

phase in morphogenesis involves more specificity both in temporal and spatial expression patterns and in the specificity of the ligands for cell surface receptors. Examples of the higher, vascular-specific growth factors include the angiopoietins and the VEGFs. Regarding the vascular system, there is little or no specificity and considerable redundancy in the genetic program for signal transduction, cell cycle regulation and apoptosis. The regulatory schemes for these cellular processes appear conserved between cell types, particularly among various epithelial cells that include the endothelium.

There are, however, compelling data to show that qualitative and quantitative expression of transcription factors are required for successful vasculogenesis (Oettgen, 2001). Current models for genetic programs suggest that not a single transcription factor, but multifactorial combinations of transcription factors in a tightly synchronized expression pattern are required for vasculogenesis.

Currently, there are little data describing a corollary to embryonic vasculogenesis in the adult. Adult tissues, however, contain "mesenchyme", stem cell populations and epithelial transformations that could produce free endothelial cell precursors similar to angioblasts. Indeed, data for "circulating endothelial cells" suggest that a CD 34 positive subset can contribute to the endothelium of sprouting blood vessels (Browning *et al.,* 1994). This population of circulating cells remains largely uncharacterized and the

mechanism whereby the cells become incorporated into the endothelium is also unknown. The experiments demonstrate that transplantation of marked cells results in incorporation of marked cells into the endothelium when angiogenesis is stimulated in the heart (Kawamoto *et al.,* 2001) or hind limb (Asahara *et al.,* 1999). The possibility of some conserved form of embryonic vasculogenesis functioning in the adult and the putative existence of a pool of endothelial stem cells is interesting and could prove therapeutically useful. Without further data on the mechanisms for how endothelial cell transplants function it is premature to speculate on a genetic program for that process.

Characterizing a genetic program for embryonic vasculogenesis relies on the results from targeted gene deletions, analysis of chimeras, *in vitro* culture of explanted embryonic tissue, and the descriptions of fate maps and tissue morphogenesis. This abundance of data shows that embryonic vasculogenesis differs from adult and embryonic angiogenesis based on the *de novo* formation of the vessels from mesodermal precursors. Therefore, comparison of the genetic programs between embryonic vasculogenesis and angiogenesis will likely show the greatest distinctions between the genes regulating mesoderm-angioblast differentiation because that is where the morphogenetic descriptions differ the most between the two processes, that is where the first major block or node exists in embryonic lethality from mutations and that is where the greatest specializations exist in regulatory molecules such as growth factors and cell adhesion molecules.

2.2
Embryonic angiogenesis

Comparison of embryonic angiogenesis and adult angiogenesis will prove difficult. Based on the current information available, a case cannot be made for a clear distinction between the two processes. Morphologically, the behavior of endothelial cells in embryonic and adult angiogenesis appears similar, i.e. extension of blood vessels from the preexisting vasculature. However, consideration of regional anatomical and physiological differences for the vasculature in the adult, consideration of different regulatory molecules for various regions and the differences between the types of capillaries and the sizes of blood vessels suggest that several differences exist in the genetic programs for adult angiogenesis depending on location and size. For example, the genetic program for coronary angiogenesis appears to rely more heavily on expression of VEGF-B than VEGF-A (Lagercrantz *et al.,* 1998; Olofsson *et al.,* 1996) and on angiopoietin-1 than angiopoietin-2 (Bi *et al.,* 1999).

It is relatively certain that these regional specializations for the genetic programs of adult angiogenesis arise during development. For example, development of closed capillaries in the blood-brain barrier versus open or fenestrated capillaries in the spleen or liver, and specialization of the high endothelial venules of the lymph system are likely components of the genetic programs. What remains unknown is whether convergent or divergent evolution is at work in developing the different programs for angiogenesis in the adult. The base, or most primitive angiogenesis regulatory pathway existing in the early embryo is yet unknown. Furthermore, would differences in the adult regulatory pathways or genetic programs diverge from this primitive pathway, or would they arise separately

and converge on similar key regulatory molecules? Some data in favor of a divergent scheme for differences in the genetic programs arise from fate mapping experiments using quail/chick chimeras (Noden, 1991; Poole and Coffin, 1989). Similar to Spemann's ectopic transplants in the amphibian (Spemann, 1938), transplant data show progressive determination where vascular grafts arising from earlier stage embryos have more potential for integration into host tissue than grafts originating from older stages of developing donors. At earlier stages the grafts are pluripotent in their ability to incorporate into virtually any tissue in the embryo, but later-stage grafts are not as successful in heterotopic transplants (Coffin and Poole, 1991). This suggests that divergent evolution is the more likely avenue for differences in genetic regulatory programs between adult tissues.

The components of the genetic regulatory program for embryonic angiogenesis contain some well-known angiogenic regulatory factors and endothelial-cell-associated proteins. Functionally they break down into the basic cellular processes mentioned above *(Figure 1)* for vascular growth and homeostasis including cell: differentiation, proliferation, apoptosis, migration, and adhesion. Like adult angiogenesis, embryonic angiogenesis is multifactorial making it very difficult to specifically block angiogenesis with a single inhibitor or through gene deletion. As mentioned above, targeted deletion of VEGF and angiopoietin family members and their receptors in null mice have produced embryonic lethal phenotypes specific to the vascular system. But the phenotypes do not suggest specific blockage of angiogenesis, but impairment of vascular morphogenesis. Those phenotypes associated, e.g. VEGF-A deletion, edema around E10 may be inhibiting the final step of angiogenesis to form a patent vasculature. But VEGF receptor data suggest the VEGFs as a family are involved in both vasculogenesis and angiogenesis by affecting cell differentiation and proliferation (Fong *et al.,* 1996; Shalaby *et al.,* 1995).

3.
The genetic program for adult angiogenesis

The cellular and molecular processes involved in adult angiogenesis are much better understood compared to embryonic angiogenesis. Because the data on embryonic angiogenesis are limited to virtually the same regulatory molecules as adult angiogenesis, it is difficult at this point to distinguish real differences between these two processes. The question remains: Is there any real difference, or has evolution simply conserved the same processes for both? Many investigators have speculated that pathological angiogenesis is similar to many other pathologies in reactivating what are essentially embryonic regulatory processes. For example, many of the previously referenced "oncogenes" turned out to be expressed in other pathologies besides cancer and to be regularly expressed in the embryo as normal, developmental regulatory genes: Hence, replacement of the term "oncogene" with more specific, appropriate terms.

There are several gene superfamilies involved in adult angiogenesis including: growth factors, cytokines, receptor tyrosine kinases, signal transduction and phosphoproteins, cell regulatory genes, cell adhesion molecules (CAMs and CADs), integrins and extracellular matrix proteins. Angiogenic inhibitors, particularly for tumor angiogenesis,

have been shown to interact with members of each one of these families to impact the cellular processes described above *(Figure 1)*. For example, farnesyl transferase inhibitors impair ras-mediated signal transduction to impair angiogenesis (Rak *et al.,* 1995) and inhibitors to VEGF and VEGF receptors limit tumor angiogenesis (Clyman *et al.,* 2002; Drake *et al.,* 2000; Ferrara and Gerber, 2001). Likewise, antibodies to integrins have been very effective in limiting endothelial cell-substrate adhesion necessary for growth and maturation of new vessels (Drake *et al.,* 1995).

The genetic program for adult angiogenesis is unique in its homeostatic function. Tumor angiogenesis, and other pathologies that stimulate angiogenesis appear to have more similarities than differences. However, regional specialization has produced differences. Brain tumors for example, are much more VEGF than FGF-2 dependent for stimulation of angiogenesis (Plate *et al.,* 1994) and differentially express integrins and cell adhesion molecules compared to other tumors (Asano *et al.,* 2000; Bello *et al.,* 2001; MacDonald *et al.,* 2001; Satoh and Kuroda, 2000; Zhou and Skalli, 2000). But the regulatory programs for homeostatic angiogenesis, especially for specialized capillaries are not well characterized. Little is know about the mechanisms for replacement of fenestrated or open capillaries, yet this process undoubtedly occurs. The most likely explanation is regional specialization that is established during development, but the regulatory genes and pathways have not been described.

4. Comparison of genetic programs for embryonic vascular development and adult angiogenesis

The single largest difference between the genetic programs for embryonic vascular development and adult angiogenesis is vasculogenesis, i.e. the precocious *de novo* formation of the initial vascular pattern in the mid-gastrulation stage embryo. With the possible exception of "circulating angioblasts" there is no equivalent in the adult, or at most adult vasculogenesis is a very *cryptic* or rare event in adult vascular homeostasis and pathology. Certainly the preferred or most conserved mode of blood vessel growth in the adult is an angiogenesis process that is morphogenically similar to the vascular sprouting form of angiogenesis in the embryo. Indeed, if we account for regional specialization of blood vessels, many of the same genes regulate adult and embryonic angiogenesis. Several reviews describe angiogenesis in very eloquent and detailed terms (Folkman 2001; Poole *et al.,* 2001). Embryonic angiogenesis lacks the mesodermal-angioblast differentiation events observed in vasculogenesis. Thus, the vasculogenesis component of the genetic program likely contains a unique subset of growth factors, transcription factors, signal transduction genes, cell-cell and cell-substrate adhesion molecules. Interestingly, very little is known about the mesoderm-angioblast differentiation events. Angiopoietins, VEGFs, ephrins and TGFβ family members have been shown to affect the remodeling process following both vasculogenesis and angiogenesis in the embryo and adult (Yancopoulos *et al.,* 2000). Models for growth factor function suggest that these growth factor "morphogens" regulate vasculogenesis, angiogenesis, and subsequent remodeling by binding to cell surface kinase receptors that trigger signal transduction, to alter

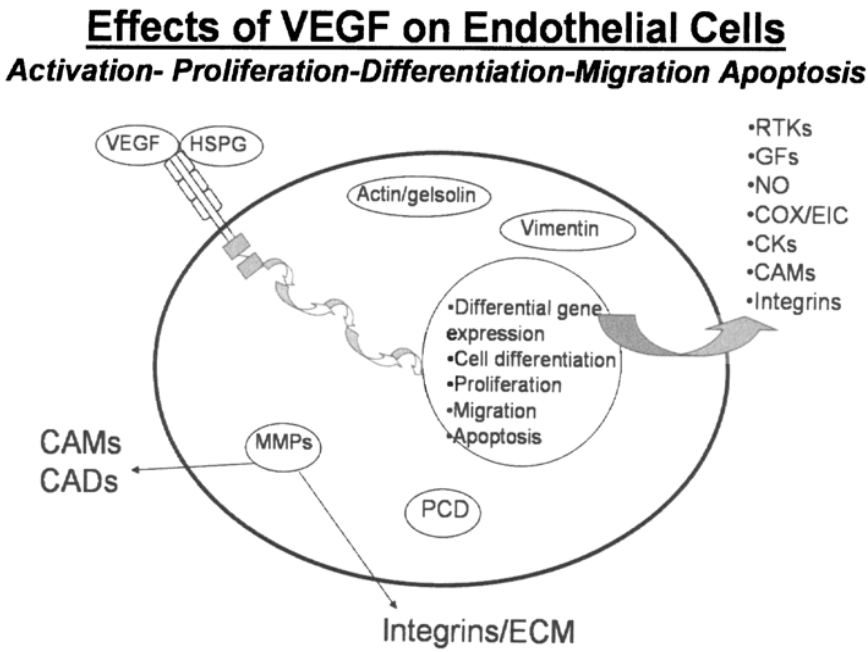

Figure 5. Effects of growth factors on the endothelium. Growth factor expression is an integral part of vascular morphogenesis. VEGF is used as an example of a heparin binding growth factor, first binding to the receptor tyrosine kinase (RTK) with a heparin sulfate proteoglycan co-factor. Ligand binding and RTK dimerization result in signal transduction (arrows), principally through kinases and phosphatases in the cytoplasm. Upon reaching the nucleus, the signal activates transcription factor expression and subsequent differential gene expression. Differential gene expression can result in several out comes for the cell including proliferation, differentiation, migration, and apoptosis (PCD). In the course of these events, growth factors can cause differential actin polymerization, intermediate filament, and matrix metalloproteinase (MMP) expression. The MMPs may cleave extracellular adhesion molecules, freeing the cell for migration. Through these mechanisms, growth factors can also "activate" quiescent endothelium for auotcrine upregulation of themselves and paracrine upregulation of RTKs, growth factors (GFs), nitric oxide (NO), cyclooxygenase (COX) and eicosanoids (EIC), cytokines, CAMs and integrins. All of these and many more molecules may be involves in vascular development and vascular homeostasis.

transcription factors to initial cell differentiation, proliferation, migration, adhesion and apoptosis *(Figure 5)*.

Based on these models, angiogenesis contains endothelial cell transitions that are absent from vasculogenesis. Following initial development through vasculogenesis or embryonic angiogenesis during development, the endothelial cell becomes bound to other endothelial cells in the monolayer and enters quiescence. Upstream events such as injury or hypoxia signal through growth factor activation *(Figure 5)* and trigger the cell to enter a new cycle of proliferation, migration and differential adhesion to extend or renew the capillary. The hypoxia-inducible factors (HIF) have been identified as key growth factor

induction genes under hypoxic conditions (Semenza, 2000). Then the endothelial cell is again bound into the monolayer to become quiescent once again. This transition, which is unique to angiogenesis (versus vasculogenesis), may occur in homeostasis or during pathologies such as tumor angiogenesis, arthritis or retinopathy. The latter component of this angiogenesis transition is similar to the differential adhesion and basement membrane production that occurs in vasculogenesis with the essential difference lying in the source of the new endothelial cell for growth and extension. Thus, it is possible that the genetic program for angiogenesis is a modification of vasculogenesis. Vasculogenesis is embryologically earlier and perhaps older phylogenetically. Therefore, angiogenesis could arise by deleting the portion of the genetic program required for mesodermal cell differentiation while adding a multifactorial, precocious process for recruiting new endothelial cells from within the monolayer. The remnants of vasculogenesis in the adult may lie in "circulating angioblasts" and other less obvious processes that are less useful than robust, simpler angiogenesis. Ultimately, this logic suggests that angiogenesis may have evolved from vasculogenesis due to the loss of mesodermal precursors as a source of endothelial cells. The genetic program for angiogenesis arises from selection of that process, through exclusion or inclusion of genes from a larger subset that regulate vasculogenesis, a more primitive process. Evolution of the genetic program for angiogenesis, therefore, involves a relative diminution of mesodermal differentiation genes and enhancement of genes for the endothelial cell transitions involved in angiogenesis. Then further evolution of the genetic program for adult angiogenesis arises for specialization of veins versus arteries, large versus mircovessels, and functional specialization of microvessels such as barriers and fenestrations.

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11
Mapping human vascular heterogeneity by *in vivo* phage display

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1. Introduction

1.1 *What is the vascular map?*

Until recently, molecular diversity of the vasculature remained largely unexplored. In the past, the circulatory system had been assumed to consist of vessels that serve a universal transport function throughout the organism and that are, therefore, morphologically and biochemically undistinguishable in different organs. Lately, it has become clear, however, that the morphology of microvessels can vary drastically among different tissues. Capillary shape and the content of supporting cells (pericytes) in pathological (tumors), as well as in physiological (adipose tissue) conditions, may drastically differ from those in other tissues. Recently, it has become apparent that vascular beds of different organs can be distinguished at the molecular level. This variability results in tissue-specific differences in binding of circulating molecules and cells to the endothelium. Thus, the combination of anatomical context, tissue-specifically expressed endothelial receptors, and the surrounding extracellular matrix creates unique microvascular environments among different organs. *Vascular mapping* involves identification and characterization of such variables, termed vascular addresses, which, as discussed below, often turn out to be endothelial cell surface proteins that show tissue-specific expression patterns. A proteome map of the circulatory system is much awaited because characterization of endothelial receptors will help to understand how specific circulating molecules, cells, or pathogens are directed to individual organs. Characterization of vascular addresses or "zip codes" will also provide the means for unraveling the molecular mechanisms of vascular disorders and for targeting therapies to specific organs (Arap *et al.,* 1998a; Folkman, 2002; Narasimhan, 2002; Rubin and Tall, 2000).

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2. Evidence for vascular heterogeneity

The circulatory system includes blood and lymphatic vessels. In each of these physiologically distinct types of vasculature, vessels are lined with a layer of endothelial cells that make contact with the fluid. Based on the functional divergence of blood and lymphatic circulation, it has been proposed that endothelial cells in these two systems may express different cell surface molecules (Karkkainen *et al.,* 2002). There is accumulating, supporting evidence, although a reliable marker completely specific for lymphatic endothelium has not yet been found. One of the receptors overexpressed by lymphatic but not blood vessels is vascular endothelial growth factor-3 (VEGF-3), however, it is also expressed by, at least some, blood capillaries during angiogenesis (Partanen and Paavonen 2001; Sleeman *et al.,* 2001). Other cell surface molecules selectively expressed by lymphatic endothelium are the lymphatic vessel endothelial hyalonuran (HA) receptor (LYVE-1) that binds HA (Jackson *et al.,* 2001), as well as desmoplakin and podoplanin (Sleeman *et al.,* 2001). The usefulness of these molecules as lymphatic markers is currently under verification.

Evidence for tissue-specific endothelial markers in blood vessels has come from studies in various areas of biology. Originally, it was discovered that expression of specific receptors in the endothelium (e.g. endothelial leukocyte adhesion molecule-1, ELAM-1) is induced during inflammation (Bevilacqua *et al.,* 1989). Indirect evidence to the apparent specialization of blood vessel surfaces in different organs is that circulating cells home to specific tissues. It has been long known that different types of tumors metastasize to specific sites. The primarily accepted explanation to this phenomenon is defined as the "seed and soil" theory, which proposes tumor type-specific cell surface markers being anchored by tissue-specific vascular receptors (Fidler, 2001). The search for proteins responsible for tumor cell homing has identified various members of various adhesion molecule families as candidate endothelial receptors involved in metastatic spread. These include integrins, cadherins, selectins, proteoglycans and immunoglobulins (Pauli *et al.,* 1990; Tang and Honn, 1994).

Preferential homing of leukocytes to the sites of inflammation also indicates that tissues contain vascular markers. It has been shown that naive lymphocytes home to secondary lymphoid organs, whereas memory/effector lymphocytes home to peripheral organs such as skin and mucosa (Burastero *et al.,* 1998; Fabbri *et al.,* 1999). As for metastatic tumor cells, several homing receptor candidates have been reported, including intracellular adhesion molecule-1 (ICAM-1), Pselectin, and α4 integrin (Biedermann 2001; Sleeman *et al.,* 2001). These receptors appear to bind ligands selectively expressed by subclasses of lymphocytes: CD-18, CD15-E, and vascular cell adhesion molecule-1 (VCAM-1), respectively (Biedermann 2001; Sleeman *et al.,* 2001).

Finally, hematopoietic stem cells home to the activated endothelium of bone marrow in response to stromal cell-derived factor-1 (SDF-1), a chemokine secreted by the bone marrow stroma (Mohle *et al.,* 1999). In this case, binding of hematopoietic progenitors appear to occur via E-selectin ligand (ESL-1) and Pselectin glycoprotein ligand (PSGL-1)

(Voermans *et al.,* 2001). These adhesion molecules bind to E-selectin expressed by bone marrow endothelial cells (Voermans *et al.,* 2001).

3. Tissue-specific vascular markers

Evidence for endothelial heterogeneity has motivated the development of methodologies for systematic identification of tissue-specific human vascular markers. The Genome Project (Lander *et al.,* 2001; Venter *et al.,* 2001) has greatly accelerated progress towards identification of human genes differentially expressed in the vasculature. Systematic analysis of human gene expression may eventually generate a vascular genome map. Technological advances have initiated numerous efforts to construct the human vascular map using nucleic acid arrays (Armstrong *et al.,* 2002; Chen *et al.,* 2001) or serial analysis of mRNA expression (SAGE) (St Croix *et al.,* 2000). An important milestone was the identification of a number of genes that are differentially expressed in the tumor endothelium, but not in normal endothelium (St Croix *et al.,* 2000). However, approaches that profile gene expression feature an important drawback, which is their current failure to address the molecular heterogeneity of blood vessels at the protein level. A large number of proteins differentially present or differentially modified in the context of individual organs will be missed by methods that measure mRNA expression.

As technologies related to proteomics advance, new approaches for systematic molecular analysis of endothelium at the protein level are arising (Cahill 2001; Peale and Gerritsen 2001). Ultimately, high-throughput profiling of protein expression will lead to the "proteome", a protein-based fingerprint, for each tissue in humans and other species. However, methods for systematic protein expression profiling may also easily overlook potential targets for intervention. These methods often do not take anatomical context into account, whereas some clinically important endothelial proteins may be expressed in restricted locations in blood vessels. Therefore, for generating a vascular map of markers, that can be used for targeting therapeutics, information derived from conventional protein profiling approaches should be enhanced by integration with data from functional screening for vascular addresses.

4. *In vivo* phage display

Our group has developed *in vivo phage display,* a functional peptide selection method initially established in rodents. In this method, peptides (displayed on M13 phage) that home to specific organs are recovered after intravenous administration of a phage display random library (Kolonin *et al.,* 2001; Pasqualini *et al.,* 2000a). Peptides homing to selective vascular beds *in vivo* were first reported in 1996 (Pasqualini and Ruoslahti, 1996). Since then, various peptide libraries have been screened for ligands selectively homing to a number of murine tissues, including brain and kidney (Pasqualini and Ruoslahti, 1996), lung, skin, pancreas, intestine, uterus, adrenal gland and retina (Rajotte *et al.,* 1998), muscle (Samoylova and Smith, 1999), prostate (Arap *et al.,* 2002a), and breast

(Essler and Ruoslahti, 2002). This strategy also revealed a vascular address system that allows tissue-specific targeting of angiogenesis-related molecules in tumor blood vessels (Arap *et al.,* 1998b; Burg *et al.,* 1999; Ellerby *et al.,* 1999; Koivunen *et al.,* 1999a; Pasqualini *et al.,* 1997). Isolation of tissue-homing peptides enables subsequent identification of the corresponding tissue-specific vascular receptors. Complementary approaches have been used to identify receptors for peptides homing to the lung (Rajotte and Ruoslahti, 1999), breast (Essler and Ruoslahti, 2002) and tumor vasculature (Bhagwat *et al.,* 2001; Pasqualini *et al.,* 2000b). Systematic implementation of this strategy will eventually lead to the construction of a complete map of mouse endothelial surface markers.

5. Data derived from mouse models and relevance to human vascular biology

A major premise for the identification of selectively expressed vascular receptors and the ligands that home to these receptors is the translation of this knowledge into the development of targeted therapeutics. Generally, coupling to homing peptides yields targeted therapeutic compounds that are more effective and less toxic than the parental compound. So far, peptides selected by homing to tumor vasculature have been used as carriers to guide the delivery of cytotoxic drugs (Arap *et al.,* 1998b), pro-apoptotic peptides (Ellerby *et al.,* 1999), metalloprotease inhibitors (Koivunen *et al.,* 1999a), cytokines (Curnis *et al.,* 2000), fluorophores (Hong and Clayman 2000), and genes (Trepel *et al.,* 2000) in mouse models.

Potentially, the information on homing ligands and their receptors in mice (Arap *et al.,* 1998b; Burg *et al.,* 1999; Ellerby *et al.,* 1999; Koivunen *et al.,* 1999a; Pasqualini and Ruoslahti 1996; Pasqualini *et al.,* 1997, 2000a, 2000b; Rajotte and Ruoslahti 1999; Rajotte *et al.,* 1998) could be used for development of targeted drugs to treat human diseases. However, it has recently become apparent that data derived from rodent models are often not easily translated into real clinical applications. Failure of mouse-generated anti-cancer drugs in human clinical trials provides a good illustration to this notion (Coussens *et al.,* 2002; Nicol *et al.,* 2001). Inapplicability of the results from the murine models to human biology may often be due to the drug not reaching its corresponding target when in the human bloodstream. Cross-species variation in expression or presentation of tumor markers, which is currently being explored, could easily account for that phenomenon. The prostate-specific membrane antigen, PSMA, provides an example of such species-specific differences. Whereas human PSMA is upregulated in the prostate and tumor vasculature, it is undetectable in the mouse prostate (Bacich *et al.,* 2001) or tumor blood vessels in the mouse (W.D.W.Heston, personal communication). Another striking example is the gene TEM7, which, in the human, is highly and specifically expressed in the endothelium of colorectal adenomas (St Croix *et al.,* 2000), whereas in the mouse it is not expressed in tumors, but instead expressed in Purkinje cells (Carson-Walter *et al.,* 2001). These findings suggest that unique protein expression patterns within endothelium—at different tissue sites, levels, or times—will account for

the predicted differences between the vascular maps being constructed for humans and mice.

The growing list of compounds generated to be beneficial in mouse tumor models and being non-potent or harmful in the human warns that, similarly to other classes of drugs, organ-homing peptides previously identified using the murine model (Arap *et al.,* 1998b; Pasqualini and Ruoslahti 1996; Rajotte *et al.,* 1998) may undergo unexpected localization in humans and, therefore, be inappropriate for targeting therapeutics. Thus, all *in vivo* phage display data derived from animal models must be carefully validated before being applied to humans. In theory, peptides identified in animal models may be validated for applicability to the human system by various *in vitro* methods, especially if the corresponding receptor is known. Peptide binding can sometimes be validated using tissue sections or tissue arrays (Mousses *et al.,* 2002). However, possible technical limitations in the evaluation of cross-species conservation of homing patterns of vascular-targeting peptides underline the necessity of designing the approaches to directly profile vasculature in humans.

6. *In vivo* phage display screening in humans

We reasoned that screening peptide libraries in humans by phage display would enable direct establishment of human vascular targeting probes. Simultaneously, vascular targeting in humans could also serve to validate previously isolated mouse vascular targeting probes potentially useful for directed delivery of therapeutic and imaging agents. In a recent report (Arap *et al.,* 2002b), we describe the first step towards this goal. A 48-year-old male patient formally declared a brain dead human subject (Wijdicks *et al.,* 2001) was enrolled in a clinical protocol involving an *in vivo* phage screening using methodology previously optimized in mice (Koivunen *et al.,* 1999b, 1999c; Pasqualini *et al.,* 2000a). The patient received an intravenous infusion of 10^{14} transducing units (TU) of an unselected phage library that displayed 7-amino-acid-long peptides (CX_7C). Shortly after infusion, biopsy samples were collected from various tissues and phage-peptides were recovered and analyzed.

To analyze library distribution in the bloodstream, we performed analysis of 4,716 amino acid sequences of the peptides recovered from five organs: bone marrow, white fat, skeletal muscle, prostate, and skin, and for each organ identified frequently isolated tripeptide motifs. Tripeptide motifs were chosen for the peptide insert analysis because a stretch of three amino acids appears to provide the minimal framework for structure formation and protein-protein interaction (Pasqualini *et al.,* 2000a). Examples of biochemical unit recognition and binding of ligand motifs to their receptors include RGD to integrins (Arap *et al.,* 1998b; Pasqualini *et al.,* 1997), NGR to aminopeptidase N/CD13 (Arap *et al.,* 1998b; Pasqualini *et al.,* 2000b), and GFE to membrane dipeptidase (Arap *et al.,* 1998b; Pasqualini *et al.,* 2000b; Rajotte and Ruoslahti, 1997; Rajotte *et al.,* 1998). We developed a character pattern recognition program to automate analysis of the CX_7C peptide sequences derived from high-throughput phage screenings. The program uses SAS (version 8, SAS Institute) and Perl (version 5.0) to conduct exhaustive residue sequence

counts in both directions and calculates relative frequencies of all tripeptide motifs encountered in the CX_7C peptides in each target tissue (or in the unselected library). To identify the motifs that were enriched in the screen, the count for each motif within a target tissue was compared with the count for that motif within the unselected library. Enrichment of certain motifs within each tissue was assessed using Fisher's exact test (one-tailed) and was considered statistically significant at $P<0.05$. Comparisons of the motif frequencies in target organs relative to those in the unselected library, as well as in other organs, indicated the nonrandom nature of the phage library distribution. Of the 25 motifs selected in the screen, 11 were enriched in a single organ, whereas the others were enriched in multiple organs. This is consistent with some of the peptides binding to tissue-specific endothelial markers and others binding to ubiquitous vascular cell surface molecules. Our results show that there is a bias in the distribution of peptide motifs to various target organs after intravenous administration of a phage library, which is striking, given that only a single round of *in vivo* screening was performed.

Based on the previous results from *in vivo* biopannings in the mouse, we predicted that some of the selected organ-specific motifs might mimic proteins that interact with differentially expressed vascular cell surface markers. For example, a peptide may mimic a ligand of a vascular receptor via a motif sufficient for receptor recognition. To identify candidate proteins mimicked by organhoming motifs, we performed computer analysis of every motif using online human protein databases. As a result, we identified 15 motifs found within previously described human proteins. Seven of the proteins containing tissue-specific motifs, were either established or putative secreted growth factors that could regulate vascular growth or homeostasis in organ-dependent manner. For example, bone morphogenetic protein 3B, a putative mimetope of which was isolated from the bone marrow, is a known growth factor that regulates bone development (Daluiski *et al.,* 2001). Similarly, interleukin 11 (IL-11), potentially mimicked by prostate-specific peptides, has been shown to signal via receptors of endothelium and prostate epithelium (Campbell *et al.,* 2001; Mahboubi *et al.,* 2000). Perlecan, potential mimetopes of which were isolated from multiple organs, is an example of a protein, which ubiquitously maintains vascular homeostasis (Nugent *et al.,* 2000). Other homologies, such as that to sortilin (Lin *et al.,* 1997) in fat, feature extracellular or transmembrane proteins that may operate selectively in the target organ.

In order to confirm that our large-scale screening approach not only identifies homing ligands, but also provides useful biological information, we showed that RRAGGS, a prostate-homing motif was a mimic of interleukin-11 (Arap *et al.,* 2002b). We demonstrated by phage overlay on human tissue sections that a prostate-homing phage displaying an IL-11 peptide mimic specifically bound to the blood vessels and to the epithelium of normal prostate, but not to control organs, such as skin. In contrast, a phage selected from the skin (displaying the motif HGGVG), did not bind to prostate tissue; however, this phage specifically recognized blood vessels in the skin. Moreover, the immunostaining pattern obtained with an antibody against human IL-11 receptor (IL-11Rα) on normal prostate tissue was undistinguishable from that of the CGRRAGGSC-displaying phage overlay. We also demonstrated the interaction of the CGRRAGGSC displaying phage with immobilized IL-11Rα at the protein-protein level

using a ligand-receptor binding assay *in vitro*. Binding of phage-CGRRAGGSC to IL-11Rα was inhibited by the native IL-11 ligand in a concentration-dependent manner. Interestingly, serum IL-11 seems to be elevated in a subset of prostate cancer patients (C.J.Logothetis *et al.,* unpublished data). Also, the expression of IL-11Rα in tumors is upregulated in some cases of human prostate cancer (M.G. Kolonin *et al.,* unpublished data). Thus, the results of human biopannings may have short-term clinical relevance.

7. Conclusions

The first human *in vivo* phage display screen clearly generated significant biological information. Data from the studies anticipated to follow this work will help to improve our understanding of the outputs of such *in vivo* library screens. Implementation of advanced software for high-throughput statistical analysis of large-scale phage display screens would be highly beneficial for this area of proteomics. In parallel, the methods for quick and efficient validation of peptide homing should be developed.

The construction of the human vascular map will expand the knowledge related to the apparent race-dependent and possibly individual-dependent differences in the profile of endothelial cell surface molecules (Wu *et al.,* 2001). Moreover, the endothelium in some organs, such as skin, can undergo alterations during inflammation in response to foreign antigens (Pober *et al.,* 2001). Homing patterns of lympocytes can switch in response to molecular changes that endothelial cells undergo in the inflammatory bowel disease (Salmi and Jalkanen, 1998). Taking this into consideration, it is the targets uncovered in our study that are likely to be valuable in multiple contexts. Exploiting this experimental paradigm systematically will allow us to carry out a molecular mapping of human vasculature in health and disease and determine the amount of overlap between the endothelium of different individuals.

Taken together, the results of the first human *in vivo* biopanning show that selective expression of vascular receptors in blood vessels can be studied with phage display directly in humans. Validation of peptide-protein homology leads identified in this project will help to determine which of the candidate peptidemimicked proteins have tissue-specifIc functions in the vasculature and to translate this knowledge into clinical applications. Development of effective targeted therapies would have major implications on the progress of various areas of medicine, and heavily relies on the prospective proteome map of human vasculature, which will be generated with phage display and other genomic and proteomic methods.

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12
Heterogeneity in angiogenesis

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1.
Introduction

In the simplest sense, angiogenesis is the process by which perfusion pathway length and vessel segment number are increased within a vascular bed. In normal situations, this effective increase in vessel density delivers more blood to the tissue facilitating tissue growth and/or increased tissue activity (i.e. endocrine production and release) (Ferrara, 1999; Risau, 1997). Consequently, angiogenesis is a primary component of tissue vascularization such as occurs during development (Breier *et al.,* 1997), following an upstream occlusive event leading to tissue ischemia (Couffinhal *et al.,* 1998) or during proliferative events as seen in tissue repair (Carmeliet and Collen, 1997; Thakral *et al.,* 1979), and tumors (Folkman and Cotran, 1976). It is generally believed that interactions between vascular cells and tissue cells, primarily through paracrine activities, play an important role in the initiation and regulation of angiogenesis within a tissue (Furcht, 1986). However, considerable detail is lacking in our understanding of the angiogenesis process and vascularization as a whole. Although we know many of the factors and signals that initiate or terminate the vascularization process, it is not clear at all how new vessel segments form while maintaining a semblance to a blood vessel and progress into a functional vessel segment within a larger, vascular bed. Clearly angiogenesis is integrated with other vascularization processes such as arteriogenesis (Schaper and Buschmann, 1999), vascular remodeling (Gibbons and Dzau, 1994), adaptation (Pries and Secomb, 2000a; Skalak *et al.,* 1998), and vascular polarization (Holder and Klein, 1999) to establish a vasculature. However, the basis for this integration and the mechanisms driving vascularization are not known.

The simple phenomenon of an increase in microvessel density, belies a very complex and highly ordered biological process. Historically, angiogenesis has been described as a process involving four general events or stages: 1) the proteolysis of extracellular matrix proteins and "loosening" of the perivascular cells, 2) the outward budding, migration, and proliferation of endothelial cells, 3) tube formation, including deposition of extracellular matrix and recruitment of pericytes, and 4) vessel maturation which stabilizes the vessel structure. These characterizations arose from early morphological and deductive studies of angiogenesis. During the initial stages of angiogenesis, new vessels sprout from the

capillary and post capillary venules of the existing vessels (Ausprunk and Folkman, 1977). Structural analysis revealed that the endothelial cells comprising the capillaries "relaxed" from the normal tube structure and sprouted out from the capillary wall in the direction of the angiogenic stimulus (Ausprunk and Folkman, 1977). Early *in vitro* evidence for basement membrane dissolution included the observations that endothelial cells produce increased levels of metalloproteinases in response to angiogenic stimulants (Gross *et al.,* 1983; Kalebic *et al.,* 1983; Moscatelli *et al.,* 1980) in addition to undergoing proliferation and chemotaxis (Folkman and Klagsbrun, 1987; Stokes *et al.,* 1990; Zetter, 1988). Interestingly, proliferation was shown to be non-essential in the early events of angiogenesis, however the continued extension of new vessels depended upon endothelial cell proliferation (Sholley *et al.,* 1984). This observation highlights the critical role migration plays in angiogenesis. Early evidence for the later stages of angiogenesis included induction of tube formation in endothelial cells in a 3-D matrix (Montesano *et al.,* 1983), endothelial cell matrix synthesis (Ausprunk, 1982; Gospodarowicz and Ill, 1980; Madri *et al.,* 1988) and growth arrest of endothelial cells by pericytes and smooth muscle cells (Orlidge and D'Amore, 1987). This broad spectrum of cellular activities, and the necessary integration into angiogenesis processes to produce a 3-dimensional, functional vascular extension, implies that a considerable number and variety of genes mediate the process.

This four-stage description of angiogenesis generally refers to how a new vessel arises from an existing parent vessel and has been considered to be distinct from other components of vascularization such as arteriogenesis (Buschmann and Schaper, 1999; Skalak *et al.,* 1998). However, growing evidence suggests that phenotypic heterogeneity is present within angiogenesis and suggests that there may be many, distinct pathways through which angiogenesis can occur (Detmar *et al.,* 1998; Friedlander *et al.,* 1995; Suri *et al.,* 1998; Thurston *et al.,* 1999). Recent genomic evidence begins to characterize the spectrum of genes involved in angiogenesis and the vasculature (Glienke *et al.,* 2000; Rohan *et al.,* 2000; St Croix *et al.,* 2000). All of this implies that "angiogenesis is not angiogenesis is not angiogenesis" and that vessel number is but one aspect of possibly many phenotypic outcomes of angiogenesis. In this regard, the four "core" stages occur during all angiogenesis events, but how these stages are realized during angiogenesis may vary. The importance of this, in addition to developing a basic understanding of blood vessel growth, pertains to targeting specific angiogenesis conditions for therapeutic manipulation. Attempts to stimulate or inhibit the angiogenesis process in the clinic may depend on the relevant "type" of angiogenesis and thus the spectrum of molecules unique to that "type". Therefore, it is important to understand the mechanisms of this complexity in angiogenesis and examine how angiogenesis integrates with all aspects of tissue vascularization in developing working models of angiogenesis. This chapter discusses the molecular (as genes) and phenotypic heterogeneity of angiogenesis in an effort to provide a more comprehensive foundation from which to study angiogenesis and vascularization.

Genetics of Angiogenesis, edited by James B.Hoying

2.
Complexity of angiogenesis

2.1
Differing molecular pathways to angiogenesis

Traditionally, a description of the angiogenesis phenotype has centered around increases in microvessel density within a tissue. In this respect, the assessment of microvessel density or content within a tissue section continues to be a primary approach to determine the extent of angiogenesis in many models (Kenyon *et al.,* 1996). Indeed, the formation of new vessel elements from existing vessels is the hallmark of angiogenesis and implies that a net increase in microvessel number is a common consequence of all angiogenesis processes. However, it is becoming evident that the morphological and functional characteristics of the vessels produced following angiogenesis can vary dramatically. Recent studies involving the over-expression of vascular endothelial cell growth factor, angiopoietin 1, or both in the skin of mice highlight this point (Detmar *et al.,* 1998; Suri *et al.,* 1998; Thurston *et al.,* 1999). In these elegant studies, transgenes were over-expressed in the skin using a promoter derived from the keratin 14 gene. Driven by this skinspecific promoter, over-expression of either VEGF or ANG1 produced increased vascularity within the ear skin of the mice. This elevated vascularity was, in part, due to an increase in microvessel number per area of ear skin indicating that both factors induced angiogenesis when over-expressed (Detmar *et al.,* 1998; Suri *et al.,* 1998). However, morphological and functional differences were present between the two resulting vasculatures. Over-expression of VEGF produced capillary-like, tortuous vessels (Detmar *et al.,* 1998; Thurston *et al.,* 1999), while ANG1 overexpression resulted in larger diameter, many-branched microvessels (Suri *et al.,* 1998). Functionally, the transgenic vasculatures differed as well. VEGF-generated microvessels were hyperpermeable and leaky, whereas the microvasculature produced by over-expression of ANG1 were not. Interestingly, over-expression of both factors generated yet another morphologically distinct microvasculature that was not leaky (Thurston *et al.,* 1999). In addition to the insight into the mechanisms by which these two factors influence the angiogenesis process, these studies suggest that, depending on the combination of factors present, the angiogenesis outcomes can differ significantly. In other words, over-expression of either VEGF or ANG1, as might occur in pathologies, induces angiogenesis and generates increased vessel number. However, the resulting vasculatures are distinct leading to different physiological conditions.

These studies reflect earlier work demonstrating that angiogenesis can occur through multiple, yet separate molecular pathways (Friedlander *et al.,* 1995). In either the corneal angiogenesis assay or the CAM assay, FGF2-or VEGF-induced angiogenesis, as indicated by an increase in vascularity. However, the neovascularization induced by FGF2 was $\alpha v\beta 3$ integrin-dependent, while the VEGF-induced neovascularization was $\alpha v\beta 5$ integrin-dependent (Friedlander *et al.,* 1995). The differential dependence on integrins between the two angiogenesis responses indicates that different molecular species (e.g. integrin pairs) can perform the common tasks (e.g. cell-matrix interactions) of angiogenesis. From

this study, it is clear, that FGF2 and VEGF can induce the utilization of two different integrin subunits (β3 and β5, respectively) during angiogenesis. And that these two growth factors, along with the sets of molecules involved in their respective pathways, induce angiogenesis. However, it is not clear whether the resulting microvessels formed in response to the two growth factors differ structurally and functionally. These aspects of the neovasculatures were not examined in the above-described study (Friedlander *et al.,* 1995). However, vessels formed in the presence of VEGF are leaky and permeable (Dvorak *et al.,* 1995), but not so in the presence of FGF2, suggests that functional differences do accompany the different molecular systems recruited by these distinct angiogenesis factors.

In the studies discussed in this section, evidence indicates angiogenesis can occur *in vivo* through multiple pathways. However, the angiogenesis examined in these studies was induced by either the over-expression or delivery of excess angiogenesis factors, reflecting a potentially artefactual pro-angiogenesis environment. Thus, it is not known if the angiogenesis process occurs through separate molecular pathways in more physiological settings. Numerous knockout mice exhibit vasculatures of varying function and character, suggesting that different angiogenesis events occur during development of these genetically altered mice. However, the vascular phenotypes in many of these mice most likely reflect defects in the angiogenesis process rather than angiogenesis occurring through different pathways. An example exception is a mouse, lacking both angiopoietin 1 and Tie 1, that does not properly develop the right-hand side of the venous vasculature, while the left-hand side remains intact (Loughna and Sato, 2001). This suggests that two distinct molecular pathways exist for left-hand and right-hand side vessels of the circulation (Loughna and Sato, 2001). In many pathological settings, particularly tumor growth, high levels of growth factors are often present (Folkman, 1990; Nguyen *et al.,* 1994). Thus tumor angiogenesis may provide a means by which to explore potential angiogenesis heterogeneity. Indeed, different tumors respond differently to anti-angiogenesis inhibitors as well as display differences in vascular surface markers (Kolonin *et al.,* 2001; Rajotte *et al.,* 1998). However, regional environmental heterogeneity within most solid tumors (Bhujwalla *et al.,* 2001; Gillies *et al.,* 1999) may make a directed and thorough examination difficult. Generating direct evidence for multiple pathways in the generation of new vessel elements via angiogenesis will require a more systematic examination of the structural and functional aspects of angiogenesis, in addition to assaying for vessel density, in a variety of angiogenesis conditions.

2.2
Neovascularization

Secondary to (or perhaps coincident with) the formation of new vessel elements during tissue vascularization is the remodeling and adaptation of neovessels into functional vessel elements of a vascular tree (i.e. arteries, arterioles, capillaries, etc.) (Buschmann and Schaper, 1999; Langille, 1993). Furthermore, new vessels, and in particular microvessels, must acquire tissue-specific features to enable proper tissue function. For example, venules become capable of mediating inflammatory events (Thurston *et al.,* 2000),

endothelial cells in glomerular capillaries are fenestrated (Esser *et al.,* 1998), while endothelium in the brain microcirculation is an impermeable, continuous blood-tissue barrier (DeFouw and DeFouw, 2000; Risau *et al.,* 1998). Whether the angiogenesis program is distinct and separate from the pathways leading to vessel differentiation and network remodeling is not clear. Traditionally, angiogenesis and vessel remodeling are viewed as individual processes (Langille, 1993; Skalak *et al.,* 1998). Arteriogenesis, an important aspect of vascular remodeling, refers to the growth and remodeling of preexistent arterial vessels into functional collateral arteries (Ito *et al.,* 1997a). This process requires proliferation of endothelial and smooth muscle cells and can result in enlargement of both vessel length and diameter (Buschmann and Schaper, 1999). The primary initiating event of arteriogenesis in the adult appears to be an increase in fluid shear stress followed by accumulation of monocytes in the remodeling artery (Arras *et al.,* 1998; van Royen *et al.,* 2001). The monocytes then release various cytokines and growth factors which creates a localized inflammatory environment for the collateral arteries to grow (Arras *et al.,* 1998; Ito *et al.,* 1997b). Unlike in angiogenesis, in which localized hypoxia and ischemia can be a potent stimulator, arteriogenesis can occur in tissue sites with normal high-energy phosphate and oxygen levels (Carmeliet, 2000; Deindl *et al.,* 2001; Ito *et al.,* 1997a). In addition, upregulation of VEGF and hypoxia-inducible genes (e.g., HIF-1α) typically associated with angiogenesis, is not necessary for arteriogenesis (Deindl *et al.,* 2001; Hershey *et al.,* 2001).

However, there are common features between angiogenesis and arteriogenesis that suggest the distinction between the vascular processes is less definite. For example, the inflammatory and mitogenic molecules involved in arteriogenesis are often the factors that are capable of regulating angiogenesis. For instance, mice lacking the angiogenic factor TNF-α or the TNF-α receptor p55 have significantly impaired arteriogenesis (Frater-Schroder *et al.,* 1987). Other molecules implicated in both angiogenesis and arteriogenesis include bFGF, PDGF, and TGF-β1 (van Royen *et al.,* 2001). Furthermore, chronically elevated shear stress, believed important in mediating arteriogenesis, has been shown to cause capillary growth (Egginton *et al.,* 2001; Milkiewicz *et al.,* 2001). Finally, eNOS appears to be a common molecular constituent in both types of vessel growth. Collateral artery development and angiogenesis are diminished in mice lacking eNOS (Lee *et al.,* 1999; Murohara *et al.,* 1998; Silvestre *et al.,* 2000).

Molecules generally viewed as angiogenesis factors appear to also participate in determining vascular network architecture and functional states, aspects of vascularization typically believed to occur independent of angiogenesis. For example, cultured glomerular microvessel endothelial cells acquired fenestrations, morphological features of glomerular capillaries, following addition of VEGF (Esser *et al.,* 1998). In the absence of added VEGF, these endothelial cells lost the fenestrations normally present *in vivo.* In addition to affecting endothelial cell differentiation, VEGF proteins appear to participate in arteriolar and venular patterning in the microvasculature of the retina (Stalmans *et al.,* 2002). The investigators examined retinal vascular development in transgenic mice expressing only one of the three VEGF transcriptional isoforms; $VEGF_{120}$, $VEGF_{164}$, or $VEGF_{188}$ (Stalmans *et al.,* 2002). Mice expressing only $VEGF_{164}$ had a normal-appearing vasculature. In contrast, mice expressing either $VEGF_{120}$ or $VEGF_{188}$ developed a capillary

plexus, characteristic of early retinal angiogenesis activities, but exhibited defects in vascular outgrowth and microvessel patterning. Mice expressing only $VEGF_{188}$ had impaired arteriolar development (as determined by ephrinB2 expression), while mice expressing only $VEGF_{120}$ had severely defective arteriolar development and partially incomplete venular development (Stalmans *et al.,* 2002).

Another example molecular system demonstrating commonality between angiogenesis and vascular network organization is the angiopoietin and Tie receptor signaling system, believed essential for proper angiogenesis (Maisonpierre *et al.,* 1997; Sato *et al.,* 1995; Suri *et al.,* 1996). In a study examining vascular development in embryos of mice lacking both angiopoietin 1 and Tie1, an orphan receptor of the Tie family, it was demonstrated that these two molecules work in coordination to establish the right-hand side of the venous vasculature (Loughna and Sato, 2001). Interestingly, this specification for venous and arterial sides of the circulation, as well as left and right symmetry occurred at a point prior to the morphological identification of left and right in the network. This suggests that network specification and maturation begins during angiogenesis and well before angiogenesis is complete. Molecular specification of arteries and veins includes expression of the Eph-ephrin receptor:co-receptor system (Adams *et al.,* 1999). Specifically, ephrinB2 marks endothelial cells of the arterial side in the microcirculation, while EphB4 is associated with the venous side (Wang *et al.,* 1998). This expression pattern continues into adulthood and includes vascular smooth muscle cells (Gale *et al.,* 2001). Mice lacking either the ephrinB2 gene or the EphB4 gene exhibit impaired angiogenesis and vascular development (Wang *et al.,* 1998). Thus, molecules involved with a process traditionally associated with post-angiogenesis activities including vascular network organization and maturation now appear to be critical in the establishment of the vascular bed via angiogenesis. More to the point, segregation and organization into a functional, polarized vascular network occurs during angiogenesis and in its absence, produces a defective vasculature. This strongly suggests that angiogenesis and vascular network maturation, including arteriogenesis, are coupled *in vivo.* Thus, it may be necessary to consider vascularization as a continuum of vascular cell activities leading to the establishment of a functional, perfusion network of which angiogenesis and arteriogenesis represent different, but complementary, aspects of that continuum. In this regard, angiogenesis may be a means by which new vascular phenotypes can be acquired within a tissue, in addition to increasing vessel density.

Three-dimensional culture of microvessel segments isolated intact from tissue results in the establishment of a neovascular bed comprised of neovessels of uniform diameter (Hoying *et al.,* 1996). Depending on the source of microvessels, this is a spontaneous process and exhibits many of the hallmark features of angiogenesis including neovessel sprouting from existing parent vessels. The neovessels continue to grow throughout the length of the culture and acquire a simple, lowmural cell containing morphology. Interestingly, angiogenesis occurs in this system in the absence of non-vascular, tissue cells and hemodynamic stimuli. That microvessels of differing diameters and character (i.e. capillaries, arterioles, and venules) (Hoying *et al.,* 1996) are placed into the culture system form uniform diameter vessels of a fairly homogeneous character suggests angiogenesis produces a "default" vessel element in the absence of these other stimuli: a 24 μm, low-

mural cell tube which differs from the range of capillary diameters in a healthy tissue of 8 μm to 12 μm (Crandall *et al.,* 1997; Pries and Secomb, 2000b) with varying mural cell densities (Hirschi and D'Amore, 1996). Furthermore, the 3-D *in vitro* angiogenesis study implies that angiogenesis can be uncoupled from the arterio genesis/remodeling processes. However, the presence of a distinct angiogenesis program does not preclude the superimposition of the remodeling process on actively growing microvessels.

3.
Genomics of angiogenesis (angiomics)

3.1
Genes of angiogenesis

Decades of *in vitro* and *in vivo* studies have generated a long and varied list of angiogenesis-related genes. A survey of the literature identifies hundreds of known genes (and counting) distributed amongst a wide variety of functional classes. The spectrum of gene classes that initiate, inhibit, regulate and coordinate angiogenesis is large and has been previously discussed. However, it is worthwhile to mention two classes of genes involved in angiogenesis, the angiogenic factors and metalloproteases, in order to highlight an additional layer of complexity in angiogenesis; tissue-derived factors.

The earliest known angiogenesis genes were the angiogenic factors which act in an autocrine and/or paracrine fashion to affect vascular cells (Folkman and Klagsburn, 1987; Zetter, 1988). This class of genes is a broadly defined set of molecules (usually soluble) that can induce angiogenesis when administered *in vivo* and/or promote endothelial cell migration and proliferation *in vitro.* Since then, the list of angiogenic factors has expanded considerably and includes inflammatory cytokines (Iruela-Arispe and Dvorak, 1997; Isner, 1996). We now know that the "angiogenic factor" is not always a vascular cell mitogen (e.g. angiopoietin) nor is it a necessary, endogenous stimulator of angiogenesis. For example, fibroblast growth factor (FGF) was one of the first well characterized angiogenic factors (Friesel and Maciag, 1995; Wang *et al.*, 1995). Based on its ability to strongly stimulate endothelial cell migration and proliferation (Friesel and Maciag, 1995; Lindner *et al.,* 1990), induce angiogenesis when delivered exogenously (Baffour *et al.,* 1992; Laham *et al.,* 2000; Parsons-Wingerter *et al.,* 2000), mediate the angiogenesis process as an endogenous factor (Villaschi and Nicosia, 1993), and upregulate a number angiogenesis-related genes (Hata *et al.,* 1999; Stavri *et al.,* 1995), one FGF family member, FGF2, is considered an important and potent angiogenic factor. However, mice lacking all translational isoforms of FGF2 develop normally and are fertile (Zhou *et al.,* 1998) indicating intact vascular development. Furthermore, angiogenesis in ischemic retinopathy (Ozaki *et al.,* 1998) and choroidal revascularization (Tobe *et al.,* 1998) in the adult FGF2 knockout mouse also occur normally. The absence of any significant vascular defects was particularly unexpected given that FGF2 is expressed by virtually all tissues (Bikfalvi *et al.,* 1997), particularly in vessels involved in angiogenesis and remodeling (Bush *et al.,* 1998). These apparently contradictory findings with the FGF2 knockout

mouse points to a more subtle role for FGF2 in angiogenesis and vascularization. The continual presence of FGF2 in tissues argues that FGF2 is important in tissue and vessel physiology. Maybe FGF2 plays a novel role in vascularization, perhaps as a co-factor in angiogenesis or a modulator of the vessel responses during the angiogenesis process. In a model of ischemic revascularization, the absence of FGF2 leads to the loss of a functional blood flow reserve in the newly formed vasculature (Sullivan and Hoying, unpublished observation) supporting this hypothesis. The FGF2 example highlights the complexity in angiogenic factor action in the *in vivo* environment and suggests that the addition of a factor can promote angiogenesis, but that does not mean that it is required for angiogenesis. This makes it particularly difficult to identify central, endogenous effectors of angiogenesis.

Another well-studied class of angiogenesis-related genes is the matrix proteases including the secreted and membrane-bound metalloproteinases (MMP) (Brignac *et al.,* 1999). MMPs are a large family of secreted and membrane-associated zincdependent extracellular endopeptidases, which degrade extracellular matrix at physiological pH (Nagase and Woessner, Jr., 1999). MMP activity is considered important in the angiogenic process not only because it establishes space for new vessel growth, but also exposes several other regulatory molecules (Clowes *et al.,* 1983; DeRisi *et al.,* 1997; Lindner and Reidy, 1995) and generates ECM fragments that are hypothesized to regulate angiogenesis (Lindner and Reidy, 1996). MMPs are produced by a variety of cells, including endothelial cells which can produce MMP-1, MMP-2, MMP-9, and MT1-MMP, all implicated in the regulation of angiogenesis (Isnerer *et al.,* 1996; Oda *et al.,* 1999; Stetler-Stevenson, 1999; Zhangef *al.,* 2002). Presumably the MMPs produced by vascular cells and tissue cells can have a similar impact on angiogenesis. For example, in a transgenic carcinoma model, proteases released and activated by infiltrating inflammatory cells induce angiogenesis within the tumors (Seymour *et al.,* 1996); activities of MMPs within the tumor released VEGF from matrix stores which in turn initiated angiogenesis (Bergers *et al.,* 2000). Finally, MMPs derived from chondroclasts rescued skeletal vascularization and ossification in MMP9-deficient mice (Vu *et al.,* 1998). From these studies, it is clear that the presence of active, secreted MMPs in a tissue, regardless of whether the proteases were released by vascular cells or the surrounding tissue cells, is sufficient to mediate angiogenesis. However, studies with MMP-deflcient mice have revealed specificity in MMP regulation of angiogenesis. MMP-2 and MMP-9 are two members of the gelatinase subfamily and even though these are distinct molecules with differences in substrate affinities, these two MMPs share many similar substrates including gelatin types I, IV, V and X and process laminin-5 (Hidalgo and Eckhardt, 2001). Mice deficient in MMP-2 develop normally and are fertile indicating that angiogenesis through development is occurring (Itoh *et al.,* 1998). However, these mice demonstrate reduced tumor-stimulated angiogenesis (Itoh *et al.,* 1998) and reduced corneal angiogenesis induced by FGF2 (Zhou *et al.,* 2000). MMP-9 null mice also develop to term and survive normally after birth (Vu *et al.,* 1998). Yet, these mice exhibit abnormal bone development related to reduced cartilage vascularization in the bone growth plate. For both of these MMP-deficient mice, normal angiogenesis occurred, as reflected by normal mouse development. However, within specific microenvironments such as cartilage involved in bone growth or a tumor, angiogenesis was limited or abnormal. This suggests

that angiogenesis in a particular local microenvironment requires specific angiogenesis regulators unique to that microenvironment. Thus, interplay and coordination of gene expression, in order to assemble the correct panel of molecules, within a tissue must be tightly regulated. An underlying implication is that there is a "core" genetic program for all angiogenesis events with a microenvironment-specific program superimposed on this core activity.

3.2
Genetic models

Genetic models, including knockout mice and zebrafish mutagenesis systems (Fishman and Stainier, 1994; Warren and Fishman, 1998), have uncovered a number of genes involved in angiogenesis, many with novel roles, and offer promise in understanding the nature of angiogenesis genetic programs. In many of these model systems, vascularization of the embryo serves as the angiogenesis "assay". Because angiogenesis is occurring in the physiological and genomic context of the embryo, the phenotypic and genetic interactions present during angiogenesis and tissue vascularization are also present. Thus, genes which have roles in coordinating multiple pathways and novel functions have been identified, which were not possible in less complicated, experimental model systems. Of course, the more complex environment of the embryo may make subsequent analysis and interpretation of findings more difficult. Nevertheless, the identification and/or characterization of a number of relevant genes including transcription factors (Dube *et al.,* 2001; Kappel *et al.,* 2000; Sato, 2000), vascular patterning genes (Adams *et al.,* 1999; Wang *et al.,* 1998; Neufeld *et al.,* 2002), angiogenic factors (Carmeliet *et al.,* 2001; Dumont *et al.,* 1998; Ferrara *et al.,* 1996; Maes *et al.,* 2002; Shalaby *et al.,* 1995), metalloproteinases (Bergers *et al.,* 2000; Itoh *et al.,* 1998; Vu *et al., 1998*), tumor suppressor genes (Haase *et al.,* 2001; Ylikorkala *et al.,* 2001), hypoxia-related genes (Carmeliet *et al.,* 1998; Dimmeler and Zeiher, 2000; Ryan *et al.,* 1998), vessel stability and specification factors (Edelberg *et al.,* 1998; Lindahl *et al.,* 1997), membrane receptors (Krebs *et al.,* 2000; Uyttendaele *et al.,* 2001), and others have resulted from transgenic-based studies.

3.3
Genome-scale gene expression

Genomic-scale studies are beginning to characterize the scope of angiogenesisand vascular-related gene expression and thus are defining the genetic programs unique to a given angiogenesis condition. Emerging genomic technologies, such as SAGE (Velculescu *et al.,* 1995) and microarrays (Duggan *et al.,* 1999) now permit a large-scale, systematic analysis of gene expression as it pertains to angiogenesis. Recent results are indicating that, as with many other developmental and adult physiological responses, there is considerable complexity in the genetic programs of angiogenesis and heterogeneity in the degree and character of vascularization. A comparison of angiogenesis in different strains of mice indicate that the extent of angiogenesis differs depending on the genetic background of the mouse (Rohan *et al.,* 2000). In this study, angiogenesis was induced in the cornea of mice

by placement of either a low (10 ng) or high (80 ng) of FGF2 source. The extent of angiogenesis differed considerably between the strains of mice with some responding minimally to the low dose treatment (i.e. C57BL/6J, C3H/HeJ, and FVB/NJ) and others exhibiting a robust angiogenesis response to both doses (i.e. 129/SvImJ, AKR/J, and BALB/cJ) (Rohan *et al.,* 2000). In addition, the different strains responded differently to angiogenesis inhibitors. Interestingly, similar responses were observed in aortic ring angiogenesis assays performed with the different strains, suggesting that the differences in angiogenesis *in vivo* were due to the microenvironment (Rohan *et al.,* 2000). Each mouse strain contains the same genes common to the mouse genome. However, polymorphisms in the genome sequences will not be shared between the different strains. As in other physiological systems, these polymorphisms are presumably affecting the extent, locale and control of gene expression within each strain (Brinkmann and Eichelbaum, 2001; Coughlin and Hall, 2002; Kottke-Marchant, 2002). Thus angiogenesis is probably not solely a function of which gene products are present, but also the proportion of each gene product and the coordination of these products with each other.

Approaches have been taken to identify genes differentially expressed by angiogenic endothelial cells. In one study, gene expression by human microvessel endothelial cells undergoing cord formation in Matrigel was compared to the same cells in a standard, 2D culture using suppression subtractive hybridization (Glienke *et al.,* 2000). Three hundred and fifty genes were identified as being differentially expressed between the two-culture conditions with some exhibiting a nearly 14-fold differential in expression as demonstrated by multiplex PCR. As expected, many of the genes observed in the Matrigel cultures were related to endothelial cell proliferation, matrix proteolysis, transcription, and signal transduction (Glienke *et al.,* 2000). In addition, a number of genes not normally associated with angiogenesis were upregulated in the cord-forming Matrigel cultures including a neurite outgrowth mediator, NrCAM, and other integrin-mediated intracellular signaling molecules. Whether these genes are expressed during angiogenesis *in vivo* remains to be determined. The predominance of matrix proteases and integrin-related gene expression in the cord-forming cultures suggests that endothelial cell-matrix interactions play a significant role in endothelial cells forming 3D structures. Further study of the identified subset of genes would provide significant insight into this aspect of angiogenesis. A second approach examining endothelial transcript profiles used serial analysis of gene expression (SAGE) to identify molecular markers of tumor angiogenesis (St Croix *et al.,* 2000). Transcripts present in endothelial cells isolated from normal colon tissues were compared to endothelial cells harvested from malignant colorectal tissues. One hundred and seventy transcripts were identified in the endothelium, with 79 being differentially expressed. Forty-six of those 79 were specifically elevated in the endothelial cells from malignant tissues (St Croix *et al.,* 2000). Following analysis of the transcripts, pan endothelial cell markers and tumor endothelial cell markers were identified and verified. Interestingly, many of these genes have unknown functions (St Croix *et al.,* 2000b). Of nine tumor-specific markers examined further, all but one (marker 8) were present in a variety of tumor vasculatures as well as in granulation tissue and the corpus luteum. The remaining marker was not observed in the corpus luteum (St Croix *et al.,* 2000), suggesting that there may be molecular distinctions between tumor angiogenesis

and physiological angiogenesis. Both of these studies are beginning to classify the gene expression programs of angiogenesis. Similar studies examining a variety of angiogenesis conditions and the progression through the angiogenesis process will start to map the genetic programs of angiogenesis and begin describing the interplay between the many genes participating in new vessel growth and vascularization.

4.
Conceptual models of angiogenesis

4.1
Balance model of angiogenesis

Classically, as new molecules involved in angiogenesis are identified, they are generally classified as either inducers or inhibitors of angiogenesis (Bussolino *et al.,* 1997). This classification is based on the idea that molecules either stimulate or facilitate the angiogenesis process or interfere with angiogenesis. For example, mice lacking a given gene would exhibit defective vascular development if that gene was promoting angiogenesis and contain hypervascular tissues if that gene inhibited angiogenesis. In this context, a model for angiogenesis has been developed based on the physiological balance between inducers and inhibitors of angiogenesis, where a loss in the regulation of this balance results in pathologically associated angiogenesis (Folkman and Hanahan, 1991; Iruela-Arispe and Dvorak, 1997; Kuchan and Frangos, 1994). In this model, the microvasculature is maintained in a quiescent, non-angiogenic state through an exquisite balance of inducers and inhibitors. Angiogenesis is initiated within a tissue when the active levels of inducers, such as angiogenic growth factors, are increased relative to the inhibitors such that the static balance is shifted in favor of angiogenesis. Similarly, a reduction in inhibitors relative to inducers will result in the same initiation of angiogenesis. For any given tissue, the nature of these inducers and inhibitors may be different, yet the balanced effect of these different factors on vessel state and angiogenesis may be the same or very similar for all tissues. The net result of the presence and interactions of inducers and inhibitors produces a given vasculature. The "tilting the balance" towards angiogenesis is often referred to as the "angiogenic switch" (Folkman and Hanahan, 1991). Best described in tumor progression, the angiogenic switch occurs at the point where the balance between inducers and inhibitors shifts to favor the inducers sufficiently to activate angiogenesis. In this regard, the tissue (or tumor) phenotype "switches" from a quiescent state to a pro-angiogenesis state (Hanahan and Folkman, 1996). The angiogenic switch is often associated with the synthesis or release of angiogenic factors. In transgenic models of fibrosarcoma, skin carcinoma and pancreatic islet carcinoma, the transition from avascular pre-malignant tumors to expanded, metastatic tumors is concomitant to the presence of active, angiogenic factors including FGF and VEGF (Bergers *et al.,* 1998; Kandel *et al.,* 1991). In both the skin carcinoma and pancreas carcinoma models, the switch to an angiogenic phenotype and carcinogenesis depends on the activity of metalloproteinases acting to release matrix bound factors

(Bergers *et al.,* 2000; Coussens *et al.,* 1999). The "balance hypothesis" and the inherent "angiogenic switch" is sufficient to explain variable changes in vessel density following angiogenesis in a variety of tissues and conditions. Transition to the corpus luteum (Stouffer *et al.,* 2001), repair of infarcted tissues (Chen *et al.,* 1994; Li *et al.,* 1996), preparation of the uterine wall for implantation (Ancelin *et al.,* 2002), and implant healing (Kidd *et al.,* 2002) can be modeled as a net increase in angiogenesis inducers. However, the balance model focuses on changes in vessel density within a tissue and does not accommodate potential differences in vessel morphology or function.

4.2
State-space model

As discussed throughout this chapter, evidence is growing that angiogenesis may provide more than a means by which to increase vessel density. During angiogenesis (or subsequent with), vessel segments can acquire different functions (i.e. leaky versus non-permeable, arterial versus venular phenotypes). These differences can be reflected in the distinct molecular pathways used during any given angiogenesis event, which may be driven by the tissue microenvironment supporting the angiogenesis activity. To accommodate these additional complexities in angiogenesis, we propose a second working model of angiogenesis called the "state-space model" *(Figure 1).* This model builds on the foundation of the balance model and incorporates these new levels of complexity. The state-space model borrows from the physical science disciplines in which multiple states are present within a given space of existence. With respect to angiogenesis, within the space of increased vessel number, there may be multiple vascular states as defined by the molecular and phenotypic characteristics. Thus, an increase in venules might be determined by an emphasis on angiopoietin 1 and $VEGF_{188}$ activities (Stalmans *et al.,* 2002; Thurston *et al.,* 1999). In regards to physiological conditions, the increase in vessel number within a tumor may be different from the increase in vessel number in granulation tissue, which may be different than the increase in vessel number in physiological angiogenesis. Each condition would represent a unique angiogenesis "state" and reflects a unique angiogenesis phenotype *(Figure 1).* A similar concept could apply to vascular regression. Although more is being learned about the mechanisms of vascular regression, we still know little about the spectrum of molecules responsible for vascular regression and the mechanisms by which these molecules bring about a decrease in vessel number. For this reason, vascular regression is modeled as a single, phenotypic process leading to reduced vessel number *(Figure 1).*

5.
Conclusions

The vasculature within a tissue serves to carry blood to and from the tissue. Virtually every tissue in the body contains blood vessels, the density and functional character of which can vary drastically. The amount of blood perfusing a tissue at any one time is a function of the number of vessels within that tissue, the extent at which these vessels are

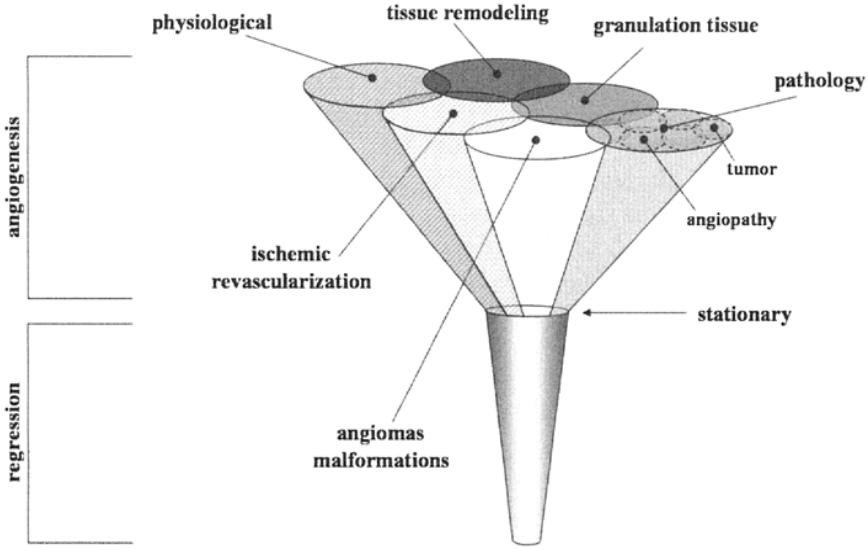

Figure 1. Schematic of state-space model of angiogenesis depicting many possible functional states for a given increase in vessel density.

patent, and the organization of these vessels into a functional perfusion network. In situations requiring an increase in perfusion (e.g. as tissue needs change) or in pathology, the relevant vascular bed can expand resulting in an increase in vascular volume. The primary mechanism driving this vascular expansion is angiogenesis. As with most cellular activities, each aspect of angiogenesis is determined by a gene expression program. However, it is becoming clear that there is no single molecular regulator of angiogenesis and that the apparently large number of molecules participating in angiogenesis must be tightly coordinated and integrated. Furthermore, the constituents of any angiogenesis process may be determined by the context within which angiogenesis is occurring. Whether that context is at the microenvironment level or at the genomic level, which determines the spectrum of genes that can be expressed and are expressed, angiogenesis can be altered to produce a variety of vascular phenotypes and activities. A better understanding of the specific coordinated networks of expressed genes and interplay of the gene products that determine the angiogenesis process would improve our ability to manipulate the vasculature and treat disease.

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